THE USE OF FLUORESCENT PROBES TO TO INVESTIGATE THE DISTANCE BETWEEN THE HIGH AFFINITY BINDING SITE AND FAD OF PYRUVATE OXIDASE

by

RICHARD O. LENHARDT

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

RICHARD O. LENHARDT

ENTITLED THE USE OF FLUORESCENT PROBES TO INVESTIGATE THE DISTANCE BETWEEN THE HIGH AFFINITY BINDING SITE AND FAD OF PYRUVATE OXIDASE

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF BACHELOR OF SCIENCE

Lowell P. Hager

Instructor in Charge

APPROVED: Lowell P. Hager

HEAD OF DEPARTMENT OF BIOCHEMISTRY

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I. INTRODUCTION

Pyruvate oxidase (pyruvate:cytochrome b_1 oxidoreductase EC 1.2.2.2) is a peripheral membrane enzyme of E. coli (1). The tetrameric enzyme requires the noncovalent binding of one FAD and one TPP molecule per monomer for activity. A divalent cation is also needed for activity (2). Pyruvate oxidase catalyzes the oxidative decarboxylation of pyruvate to acetate, CO_2, and reduced FAD (1). Reduced FAD is oxidized in vivo by a cell membrane electron transport system that includes ubiquinone-6 and cytochrome b_1 (3,4). FAD can be oxidized in vitro by electron acceptors as ferricyanide, which is used in this laboratory for routine assay purposes. The monomeric weight of the enzyme is 60,000 daltons (5).

The activity of pyruvate oxidase can be increased up to 25 fold by either mild proteolytic treatment (6) or the binding of monomeric or micellar amphiphiles (7). Proteolytic treatment leading to activation occurs when TPP, a divalent cation, and reduced FAD are present. Both the substrate pyruvate and the reducing agent dithionite can reduce the FAD group (6). Lipid activation is found when TPP, a divalent cation, and pyruvate are present with the lipid. Pyruvate oxidase demonstrates an increased affinity for lipid binding under the same conditions as for lipid activation. Flavin reduction is not necessary for increased lipid binding (8). Proteolytic nicking and lipid binding have been shown to be mutually exclusive (9). This observation has led to the hypothesis that proteolytic and lipid activation involve similar conformational changes.

This laboratory is involved in elucidating the molecular mechanism by which activation occurs. The purpose of my project was to determine the distance between the lipid binding site and the FAD cofactor. Such information should help elucidate the lipid activation mechanism.

Pyruvate oxidase contains both high and low affinity lipid binding sites. The high affinity sites are involved in enzyme activation. The low affinity binding sites have been shown to number at least 15 sites per tetramer (8). Pyruvate oxidase can be activated by both anionic and cationic amphiphiles. Since the $K_{1/2}$ of binding is lower for anionic lipids than for cationic lipids, it was hypothesized that a positively charged group is near the high affinity lipid binding site. Using carbodiimide, John White has covalently bound lauric acid to the high affinity binding sites. This technique has made it possible for a fluorescent fatty acid to be covalently bound to the enzyme. Covalently binding a fluorescent fatty acid to the high affinity sites is the first step in energy transfer experiments to determine the distance between the high affinity sites and the FAD cofactor.

Förster developed the theory or dipole-dipole energy transfer (10). Electronic excitation energy can be transferred from a fluorescent energy donor to an appropriate energy acceptor over distances as great at 70 A. The distance between the centers of the donor and acceptor can be determined by measuring the efficiency of energy transfer (11). In the case of pyruvate oxidase, the donor would be a fluorescent fatty acid and the acceptor would be FAD or an appropriate analog (FMN, riboflavin).

II. EXPERIMENTAL PROCEDURES

A. Materials

Beta-Parinaric acid (BPA), 12-(9-anthroyloxy)dodecanoic acid (ADA), and 1-pyrenedodecanoic acid (PDA) were purchased from Molecular Probes, Inc. The company has moved across the country several times and is presently located in Junction City, Oregon. Anilinonaphthalenesulfonate (ANS) was a gift from G. Weber's laboratory. Sodium dodecyl sulfate (SDS) was purchased from Pierce. Argon was obtained from Air Products and dimethyl sulfoxide (DMSO) from E.K. Industries. 2,6-ditert-butyl p-cresol (BHT), thiamin pyrophosphate (TPP), sodium pyruvate, and ovalbumin were bought from Sigma. Sodium ferricyanide was purchased from ICN Pharmaceuticals.

B. Methods

1. Purification of Pyruvate Oxidase

I have purified the enzyme three times, the last time alone. The procedure is outlined in a paper by Recny and Hager (12). Several variations from the published protocol were used:

a) HPLC was used instead of Sephadex G-200 chromatography for the last step.

b) Pyruvate oxidase was isolated from *E. coli* CG-5 after January 1983 because the W191-6 strain previously used no longer contained active enzyme.

c) The final concentration of Polymin P added to the enzyme extract was changed from 0.33% to 0.175%. The change was necessary because of the new strain used.

2. Enzyme Assays

The activity of pyruvate oxidase was assayed in 0.5 cm cuvettes at room temperature. The procedure was :

a) Mix 600 µl assay buffer

0-20 µl pyruvate oxidase

200 µl 100 µM SDS

b) Incubate six minutes

c) Add 200 µl 45 mM $Na_3Fe(CN)_6$

d) Mix and record decrease of A_{450}

The assay buffer contained:

180 µM TPP

226 mM Na pyruvate

16.4 mM $MgCl_2 \times 6H_2O$

10.2 mM Na_2HPO_4

72.5 mM $NaH_2PO_4 \cdot H_2O$

pH adjusted to 6.0

For assays using lipids other than SDS, 50 µl of the lipid in DMSO and 150 µl of water were substituted for 200 µl of SDS solution. Specific activities were calculated by the formula:

$$\text{Specific activity } (\mu\text{mole } CO_2 \text{min}^{-1}\text{mg}^{-1}) = \frac{6\ (\text{change in } A_{450}/10\ \text{sec})}{(0.218)\ (\text{ml of aliquot})\ (\text{concentration of sample in mg/ml})}$$

3. Protein Determinations

I have used four methods to quantitate protein in a sample. All values are for 1.0 cm cuvettes:

a) Concentration (in mg/ml) = 0.84 x A_{280}

This method is least accurate and was used only when the others didn't work or were not available.

b) Concentration (in mg/ml) = 1.45 x A_{280} - .74 x A_{260}

This is more accurate than the preceding method since this method accounts for nucleotide absorbance (13).

c) Concentration (in mg/ml) = 4.11 x A_{438}

This method should only be used for purified, oxidized pyruvate oxidase since it depends on the extinction coefficient of pure, oxidized enzyme (2).

d) Bradford method (14) using Bio-Rad protein dye reagent.

This is the preferred method because of its accuracy and ability to measure protein for any redox and purity state of pyruvate oxidase. With each assay an ovalbumin standard composed of duplicates should be prepared. This accounts for color changes resulting from differences in incubation time and temperature across assays.

4. Handling of Fluorescent Probes

The handling of beta-parinaric acid followed the techniques outlined by R. Fairley (15). The fatty acid was minimally exposed to air and all solutions containing the fatty acid were deaerated with argon for at least ten minutes. Deaeration procedures were not required for the handling of anthroyloxydodecanoic acid and pyrenedodecanoic acid. However, the solid samples were stored wrapped in foil, since they are light sensitive.

5. CMC Determinations

The CMC of fatty acids was determined by light scattering at 660 nm. When the light scattering rose above negligible levels, micelles were being formed.

6. Covalent Binding of Lipids to Pyruvate Oxidase

The protocol of the covalent binding reaction and the subsequent dilution assay was:

a) Mix 385 µl 2X assay buffer
363 µl 50% glycerol in water
111 µl 2.6 mg/ml pyruvate oxidase
50 µl lipid activator in DMSO
910 µl

b) Incubate six minutes

c) Add 90 µl of 7.84% (w/v) carbodiimide

d) Remove 10 µl aliquot and add to:
600 µl assay buffer
190 µl water

e) Immediately add 200 µl 45 mM $Na_3Fe(CN)_6$

f) Record decline of A_{450}

A carbodiimide control should always be prepared with the experimental sample. The control contains the same reagents as the experimental sample but for carbodiimide. 20 µl aliquots were removed instead of 10 µl aliquots for the dilution assays with BPA and PDA. The covalent binding reaction often was run at a multiple of the values given. The final concentration of the enzyme in the binding reaction varied from 0.2 mg/ml to 0.3 mg/ml. 50%

glycerol was added to give a final concentration of roughly 20% (v/v). Glycerol helps stabilize the enzyme.

7. Spectral Measurements

All absorbance spectra and most enzyme assays were measured by a Cary 219 spectrophotometer. Occasionally, a Beckman CIII spectrophotometer was used for enzyme assays. Fluorescence spectra were recorded with G. Weber's fluorometer or the MPF-44 fluorometer in R. Gennis's laboratory.

8. Attempts to Reoxidize Reduced, Lipid Bound Pyruvate Oxidase

Three techniques were tried to reoxidize lipid covalently bound pyruvate oxidase:

a) Dialysis

A 1.5 ml sample of pyruvate oxidase covalently bound to ADA and a 1.5 ml carbodiimide control were separately dialzyed against 1.0 l of 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer. After two hours, the samples were placed in 1.0 l $NaPO_4$ buffer containing 45 mM $K_3Fe(CN)_6$. After two hours in the second dialysis buffer, the samples were placed in four successive changes over 12 hours of the original buffer. The cycle was again repeated, but over 2.5 days instead of 16 hours.

b) Dithionite reduction and reoxidation with O_2

Na dithionite was added to pyruvate oxidase to reduce the enzyme. The covalent binding reaction mixture contained the appropriate amount of 2X assay buffer without pyruvate. After the covalent binding could have occurred, the sample could be bubbled with oxygen to oxidize Na dithionite and the lipid bound enzyme. All samples but the enzyme and Na dithionite solutions were deaerated with Argon.

c) Chelation of Mn^{2+}

Since Mn^{2+} is not soluble in a $NaPO_4$ solution, a 2X hepes buffer was used in lieu of the 2X assay buffer in the covalent binding reaction.

2X hepes buffer

100 μM Mn^{2+}

1.0 mM TPP

200 mM Na pyruvate

200 mM hepes

pH adjusted to 6.8

After the carbodiimide addition and a six minute incubation, 0.65 M EDTA in water was added to the covalent binding reaction mixture to give a final concentration of 70 mM. The chelator EDTA does not readily dissolve in water. Increasing the pH to 13, however, easily dissolves the EDTA. The pH can then be lowered to 8.2 while still maintaining EDTA in solution. The final concentration of the enzyme was 0.2 mg/ml in these experiments.

9. Attempts to Separate Covalently Bound and Unbound Probe

I have tried six techniques to separate covalently bound and unbound anthroyloxydodecanoic acid. The lipid:monomer ratio in all the samples containing covalently bound ADA was 2:1. The final enzyme concentration of the binding reaction was between 0.20 mg/ml and 0.32 mg/ml. The carbodiimide control was treated exactly the same as the sample with covalently bound ADA.

a) Ammonium sulfate precipitation

To a 1.8 ml sample containing covalently bound ADA, I added ammonium sulfate to give 100% saturation. After centrifuging down the precipitated

enzyme, the pellet was resuspended in 2.0 ml of 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer. After centrifuging to clarify the solution, the enzyme was again precipitated and resuspended. The temperature of the sample was always 4°C.

b) Dialysis

Three approaches to separate unbound ADA from bound ADA by dialysis were tried. The dialysis experiments were carried out using 12,000-14,000 molecular weight cutoff, standard cellulose dialysis tubing from Spectrapor. Dialysis was performed at 4°C.

The first experiment involved dialyzing a 4 ml covalent binding reaction mixture over two days against 1.0 l of 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer containing 5 x 10^{-3}% (w/v) bovine serum albumin. The binding reaction mixture had a 38:1 ADA:monomer ratio. The dialysis buffer was changed once after the first day. After the second day, the fluorescence emission spectra of the samples excited at 361 nm were recorded.

The second experiment tested the movement of ADA through the dialysis tubing. 10 ml of 3.0 x 10^{-5} M ADA in 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer was dialyzed against 4.0 l of the same $NaPO_4$ buffer for 100 hours. The dialysis buffer was changed after the second through sixth measurements of A_{367}. The buffer flask was wrapped in foil to minimze light exposure.

The third experiment was undertaken to determine whether reduced, ADA covalently bound enzyme could be reoxidized. The protocol is given in chapter IIB, section 8a.

c) Ultrafiltration

Samples containing ADA covalently bound enzyme were concentrated by an Amicon ultrafiltrator. 100,000 MW cutoff, type c ultra-filtration membranes from Nucleopore were used (XM100). 50,000 MW cutoff membranes (XM50) were

also used in the controls to test if the ADA was removed from the solution. Some samples were concentrated through more than one ultrafiltration cycle. For example, one sample was concentrated to half its original volume and buffer was added to bring the solution up to the original volume. This was repeated three times so the total dilution and concentration was 8 fold. The buffer used in diluting the samples was either assay buffer or 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer.

d) Ion-retardation resin

A 1 x 15 cm column of AG11A8 from Bio-Rad was prepared by swelling 6 g of the resin in 100 mM $NaPO_4$, 20% glycerol, pH 6.0 (5.5 for the second run) buffer. The column was washed with approximately 5 volumes of 1.0 M NH_4Cl and then 20 volumes of the same $NaPO_4$ buffer in which the resin was swelled. After loading a sample, the column was washed with the column buffer and 1.5 ml fractions were collected. The elution profile of the enzyme was monitored by A_{438} measurements, and the elution of ADA was followed by measuring the A_{367} of the fractions. Occasionally, the column was washed with a strong ionic solution, as 1.0 M NaCl or 3.0 M NaCl, after the $NaPO_4$ washes, to attempt to elute enzyme or ADA tightly bound to the column. The column was run at room temperature.

e) Extraction with organic solvents

Two ml samples of pyruvate oxidase covalently linked with ADA were extracted with six ml of organic solvent. Extraction was attempted with chloroform and heptane. The extraction was performed at 4°C in foil wrapped vials. All the samples were stirred slowly and simultaneously since a 4 place stir plate was used. After eight hours of gentle stirring the stir rate was

increased. After seven hours at an increased stir rate I shook the samples vigorously by hand in series of four shakes.

f) Sephadex G-50 column

Two columns were used. The first measured 1 x 10 cm and the second 1.4 x 24 cm. All manipulations except for swelling were done at 4°C. The resins were swelled at 90°C in water for one hour. The resins were then allowed to cool before pouring. After pouring, the column were washed with 2-5 volumes of 150 mM $NaPO_4$, 25% glycerol, pH 6.0 buffer. The samples were eluted with the same buffer. The sample size was 0.5 ml for the smaller column and 2.0 ml for the larger column. The void volumes were determined by Blue Dextran 2000 to be 2 ml and 15 ml. The elution of the enzyme was followed by A_{438} measurements and occasionally by the Bio-Rad protein assay. Elution of the assay buffer components was followed by A_{280} and the elution of ADA by A_{367} measurements.

III. RESULTS

A. Purification of Pyruvate Oxidase

The new CG-5 strain contains more than three times the total pyruvate oxidase activity than the old W191-6 strain:

Strain	Activity/dry weight of cells (μmole CO_2 evolved min^{-1}/g)
W191-6	33.5 (from reference 12)
CG-5	118. (from cells grown by G. Leisman and R. Lenhardt)

The results of my enzyme preparation using the new bacterial strain are given in table 1 and figure 1. The HPLC purification step was not used in this preparation because the enzyme was sufficiently pure. This is seen in the SDS polyacrylamide gel of the purification fractions in figure 1. The enzyme can be stored at 3-4 mg/ml in 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer at 4°C and be stable for months. At high concentrations, 10-15 mg/ml, and under the same conditions, the enzyme concentration falls to half the original in a few weeks with little change in specific activity.

B. Probe Activation of Pyruvate Oxidase

The activation profiles of four fluorescent probes are shown in figures 2 and 3. The four probes, beta-parinaric acid, anilinonaphthalenesulfonic acid, anthroyloxydodecanoic acid, and pyrenedodecanoic acid all activate pyruvate oxidase substantially more than the water and DMSO controls. The water control measures the activity of unactivated pyruvate oxidase. The DMSO control measures how much the solvent carrier of the probes, DMSO, activates the enzyme.

Table 1

Purification of Pyruvate Oxidase from the New CG-5 Strain

Purification step	Total protein (mg)	Total activity (μmole $CO_2 min^{-1}$)	Specific activity (μmole $CO_2 min^{-1} mg^{-1}$)	% Recovery
1. Crude extract	60,200	88,000	1.46	100
2. Polymin P supernate	42,200	81,200	1.92	92.2
3. Ammonium sulfate fractionation	19,700	46,700	2.36	53.1
4. Heat denaturation	3,660	39,400	10.7	44.8
5. Concentrated DEAE Sephadex fractions	188	17,800	94.6	20.2
6. Low ionic strength precipitation	68.2	7,670	112	8.7

Figure 1

SDS Polyacrylamide Gel of Purification Steps

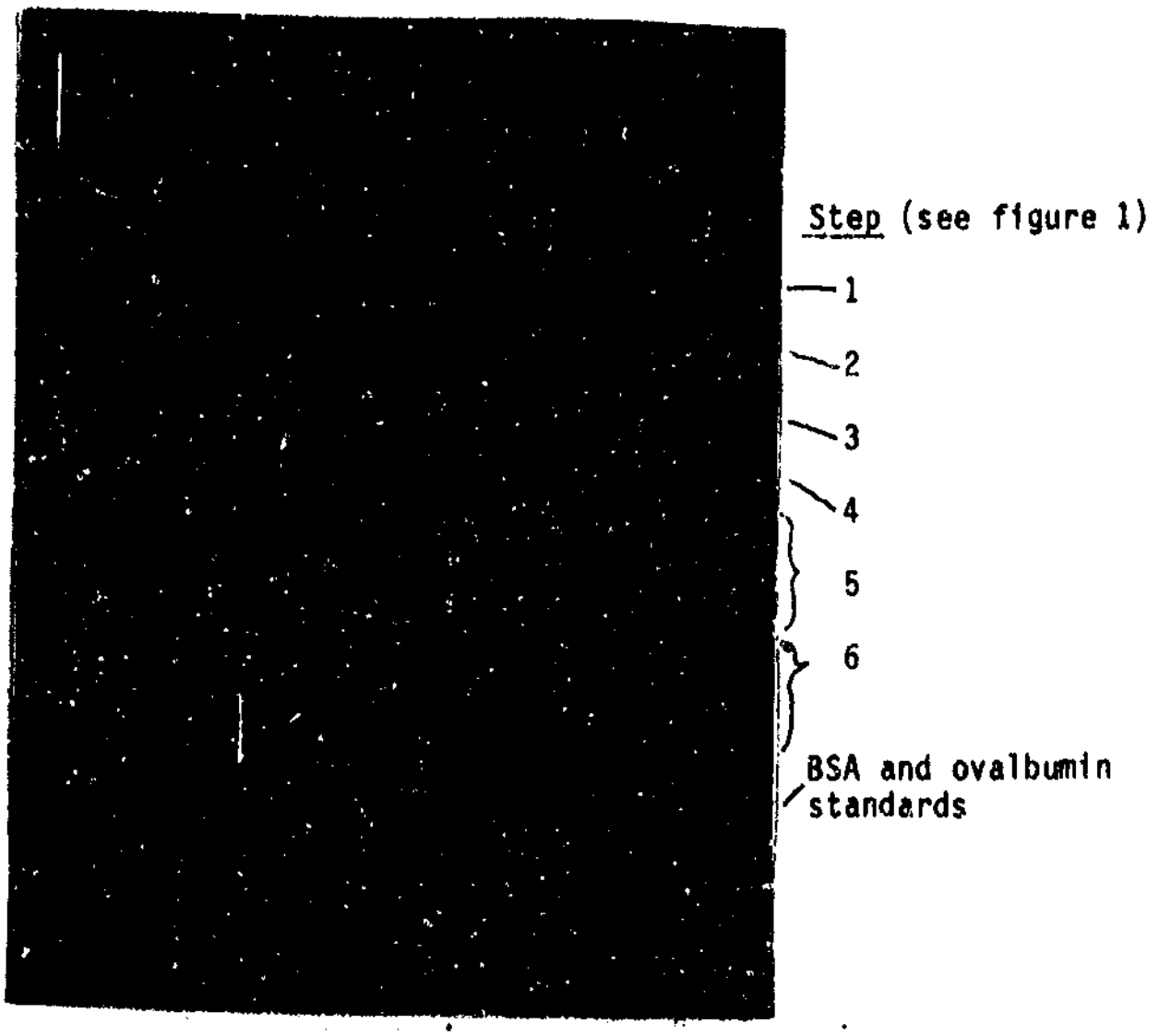


Figure 2A

ACTIVATION PROFILE OF BETA-HYDROXYBUTYRIC ACID

Activity As % of 20μM SDS Activity

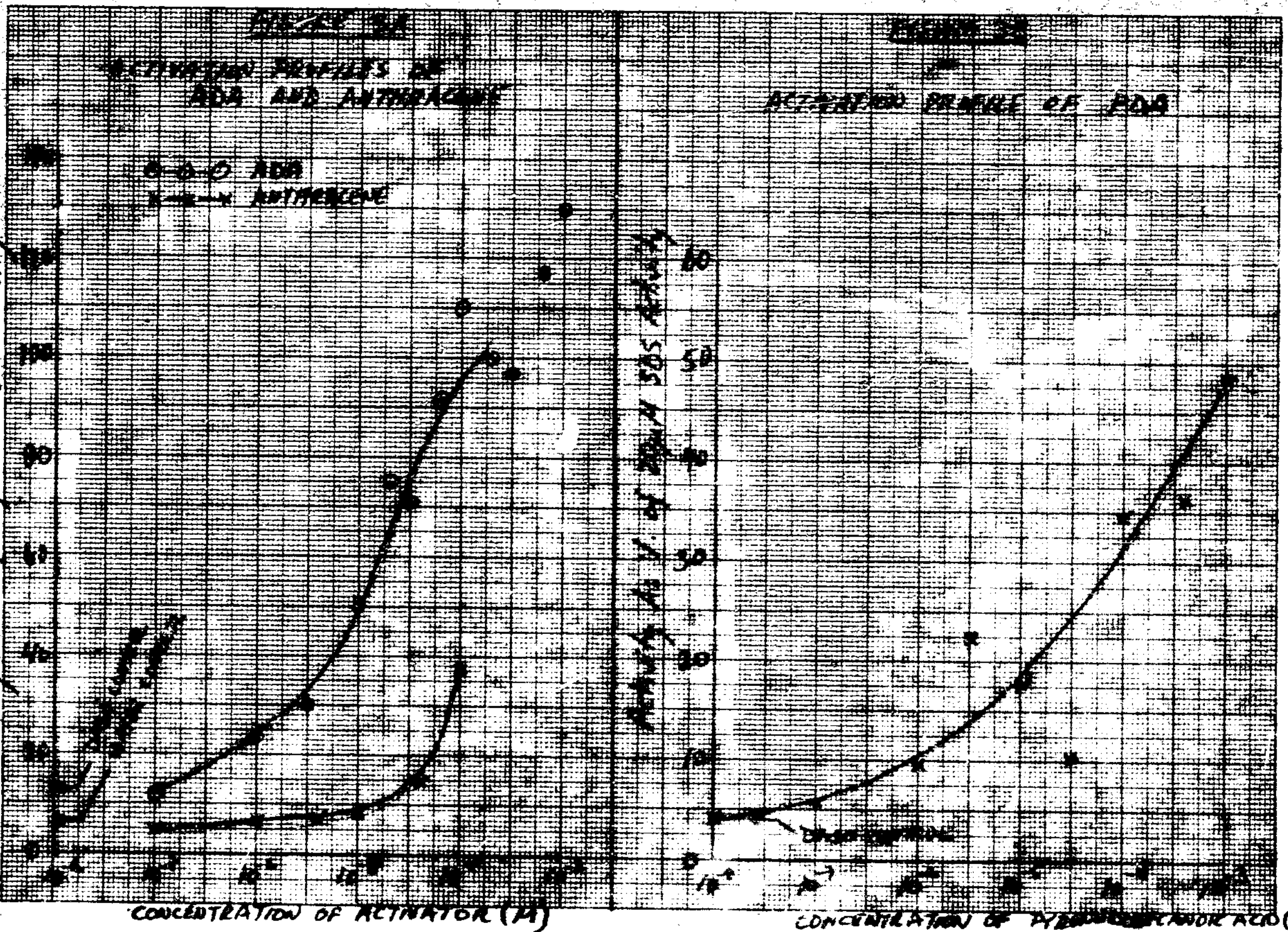
CONCENTRATION OF β-HYDROXYBUTYRIC ACID (M)

Figure 2B

ACTIVATION PROFILE OF ANS

Activity As % of 20μM SDS Activity

CONCENTRATION OF ANS (M)



Since DMSO was the probe solvent, I examined the effects of DMSO on unactivated and lipid (SDS) activated enzyme. The results shown in figures 4A and 4B demonstrate that DMSO activates unactivated enzyme and inactivates lipid activated enzyme.

C. Covalent Binding of Lipids to Pyruvate Oxidase

The covalent binding reaction with carbodiimide and the subsequent dilution assay to prove covalent binding were tried with four different lipids:

1. Beta-parinaric acid and lauric acid

I tried seven times to covalently bind beta-parinaric acid to pyruvate oxidase. The results of the dilution assays of the attempts are summarized in Table 2. It appears as beta-parinaric acid was not covalently bound to the enzyme. To test for faulty reagents or technique, I tried and succeeded in covalently binding lauric acid to the enzyme. Good evidence for covalent binding occurs when the experimental sample's activity is 200% that of the carbodiimide control's activity. Typical dilution assay time courses for BPA and lauric acid are shown in figures 5A and 5B. In the last attempt to covalently bind beta-parinaric acid to the enzyme, I pre-reacted BPA with carbodiimide before adding the lipid to reduced enzyme. The BHT concentration in the BPA in DMSO stock solutions was kept at .02% (w/v), ten times less than what is used in the literature. The concentration was reduced because BHT precipitated in the binding reaction mixture at higher concentrations. BHT is an anti-oxidant that helps stabilize BPA in solution.

Effect of
% (v/v) DMSO
on Activity of 20 μM SDS

Effect of
% (v/v) DMSO
on Activity of 20 μM SDS

Activity As % of

% DMSO (v/v) in Assay Cuvette

% DMSO (v/v) in Assay Cuvette

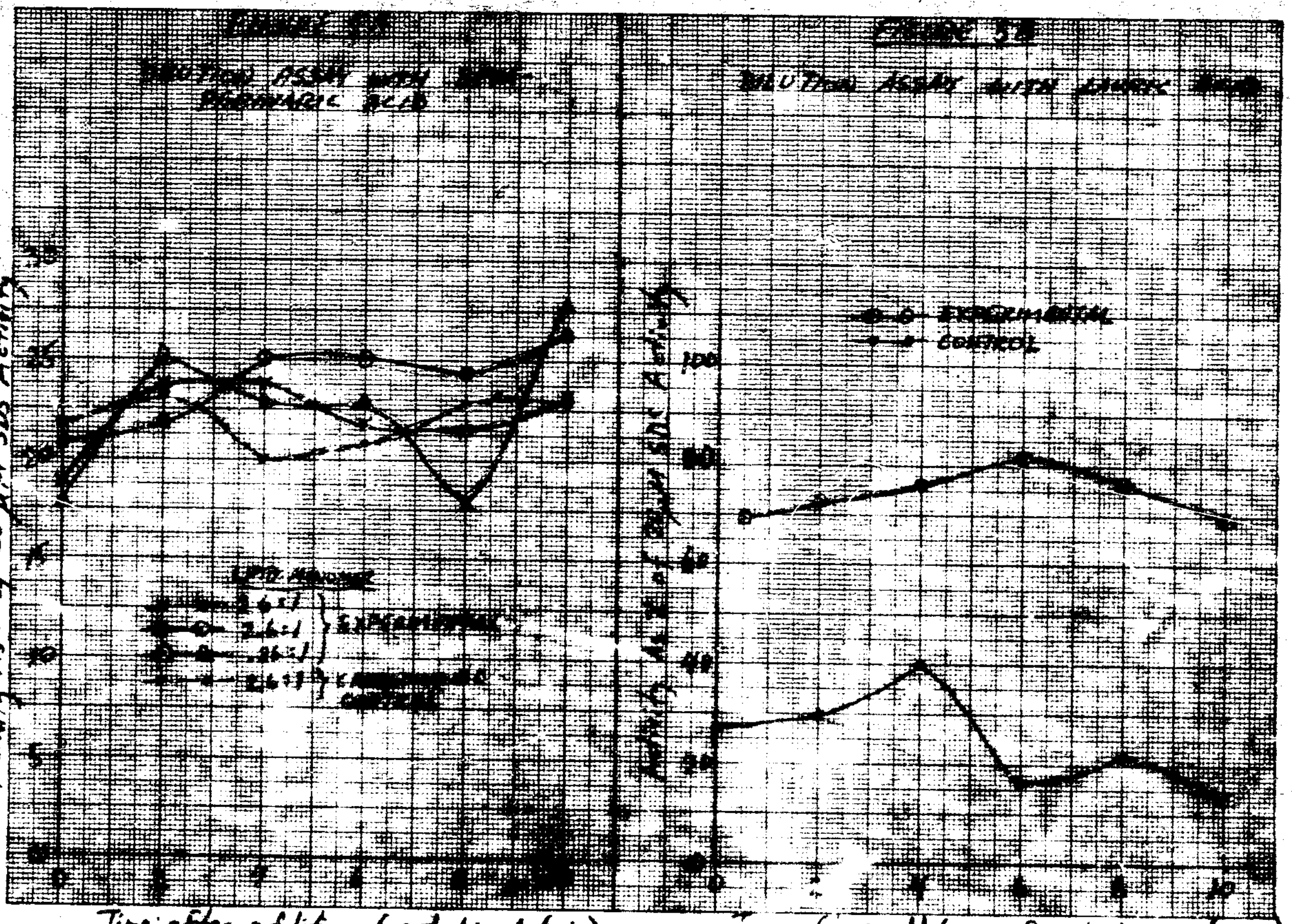


Table 2

Dilution Assays with Beta-Parinaric Acid and Lauric Acid

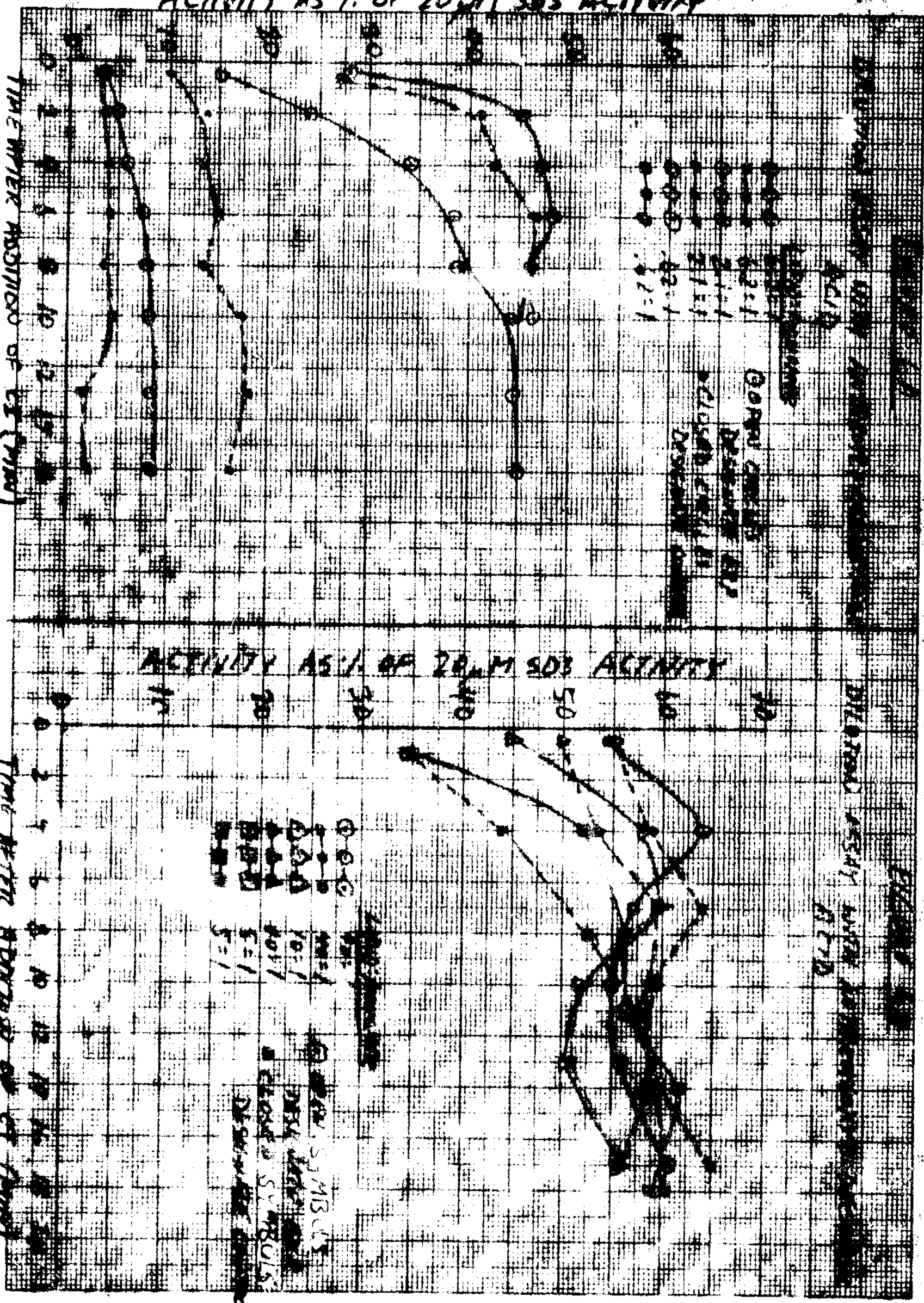
Attempt #	Lipid	Lipid concentration in reaction mixture (M)	Lipid: monomer ratio	Highest activity as % of 20 μM SDS activity in dilution assay	Activity of exp. as % activity of control
1	BPA	$5x10^{-5}$	13:1	24	100
2	LA	$3x10^{-4}$	67:1	80	250
3	BPA	$5x10^{-5}$	13:1	27	120
4	BPA	$5x10^{-5}$	13:1	27	---
5	BPA	$1x10^{-4}$	26:1	22	---
		$1x10^{-5}$	2.6:1	25	100
		$1x10^{-6}$	.26:1	22	---
6	LA	$3x10^{-4}$	67:1	64	---
7	BPA	$1x10^{-4}$	13:1	27	---
8	BPA	$1x10^{-4}$	18:1	34	100
9	BPA	$1x10^{-4}$	18:1	36	50

2. Anthroyloxydodecanoic acid

The covalent binding reaction and subsequent dilution assay were tried many times at very different lipid:monomer ratios, from below 1:1 up to 40:1. The best ratio demonstrating covalent binding was 2:1 when the monomer concentration in the reaction vessel was near $5x10^{-6}$ M. The results of the dilution assay tried at low to middle lipid:monomer ratios is shown in figure 6A. The dilution assays at higher lipid:monomer ratios is shown in figure 6B.

ACTIVITY AS % OF 20 μM SDS ACTIVITY

TIME AFTER ADDITION OF EF (min)



ACTIVITY AS % OF 20 μM SDS ACTIVITY

TIME AFTER ADDITION OF EF (min)

3. Pyrenedodecanoic acid

The dilution assay was tried twice with pyrenedodecanoic acid. The results are given in the following table:

Attempt #	Lipid concentration (M)	Lipid: monomer ratio	Highest activity as % 20 μM SDS activity in dilution assay	Activity of experimental as % activity of control
1	2.5×10^{-3}	690:1	31	115
2	1.0×10^{-3}	344:1	18	100

Thus, the results do not support that PDA was covalently bound to the enzyme.

D. Properties of Anthroyloxydodecanoic Acid

1. CMC of ADA

The CMC of ADA and lauric acid were determined by measuring the concentration of lipid at which light scattering detected at 660 nm becomes apparent. The results are:

Lipid	Solvent	CMC
ADA	assay buffer with 20% glycerol	8×10^{-5} M
ADA	water	2×10^{-4} M
lauric acid	assay buffer with 20% glycerol	3×10^{-5} M

As expected, micelles formed at a lower concentration for ADA than for lauric acid, because of the anthracene moiety on the former lipid. The light scattering with concentration of lipid is shown in figure 7A.

2. Light Sensitivity

Molecular Probes, the manufacturer of ADA, reports that ADA is light sensitive and should be stored in the dark. Also, the Merck Index (1976) reports that anthracene exposed to light dimerizes. ADA consists of anthracene covalent bound to the non-carboxyl end of lauric acid. So the light sensitivity of ADA was tested. The decline in absorbance at 367 nm over time of a 3.0×10^{-5} ADA in 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer demonstrates the light sensitivity of ADA. This is shown in figure 7B. The experiment was carried out at room temperature, and a 60 watt incandescent light source was constantly located 50 cm from the sample.

E. Attempts to Reoxidize Reduced, Lipid Bound Pyruvate Oxida

1. Dialysis

The fall of specific activity with time of dialysis for a sample of ADA covalently bound to pyruvate oxidase and the carbodiimide control is shown in figure 8B. The protein was measured by the Bio-Rad microassay. The large error bars are caused by uncertainty in protein concentrations. The spectra of the two samples after the first day of dialysis is shown in figure 9. The spectra of the samples after four days of dialysis are not much different from the spectra after the first day of dialysis. The spectral shape is similar to reduced enzyme. However, the absorbance of the solution is many times larger than would be expected with a pyruvate oxidase concentration of 0.3 mg/ml.

Figure 2A

Light Scattering at 660 mμ

X—X—X Acetate buffer
O—O—O Water in buffer
Δ—Δ—Δ Formic acid in acetate buffer

$A_{660\ mm\ path}$

(CONCENTRATION OF LIPID (M))

Figure 2B

LIGHT SENSITIVITY OF A_{660}

Time of Incubation (hours)

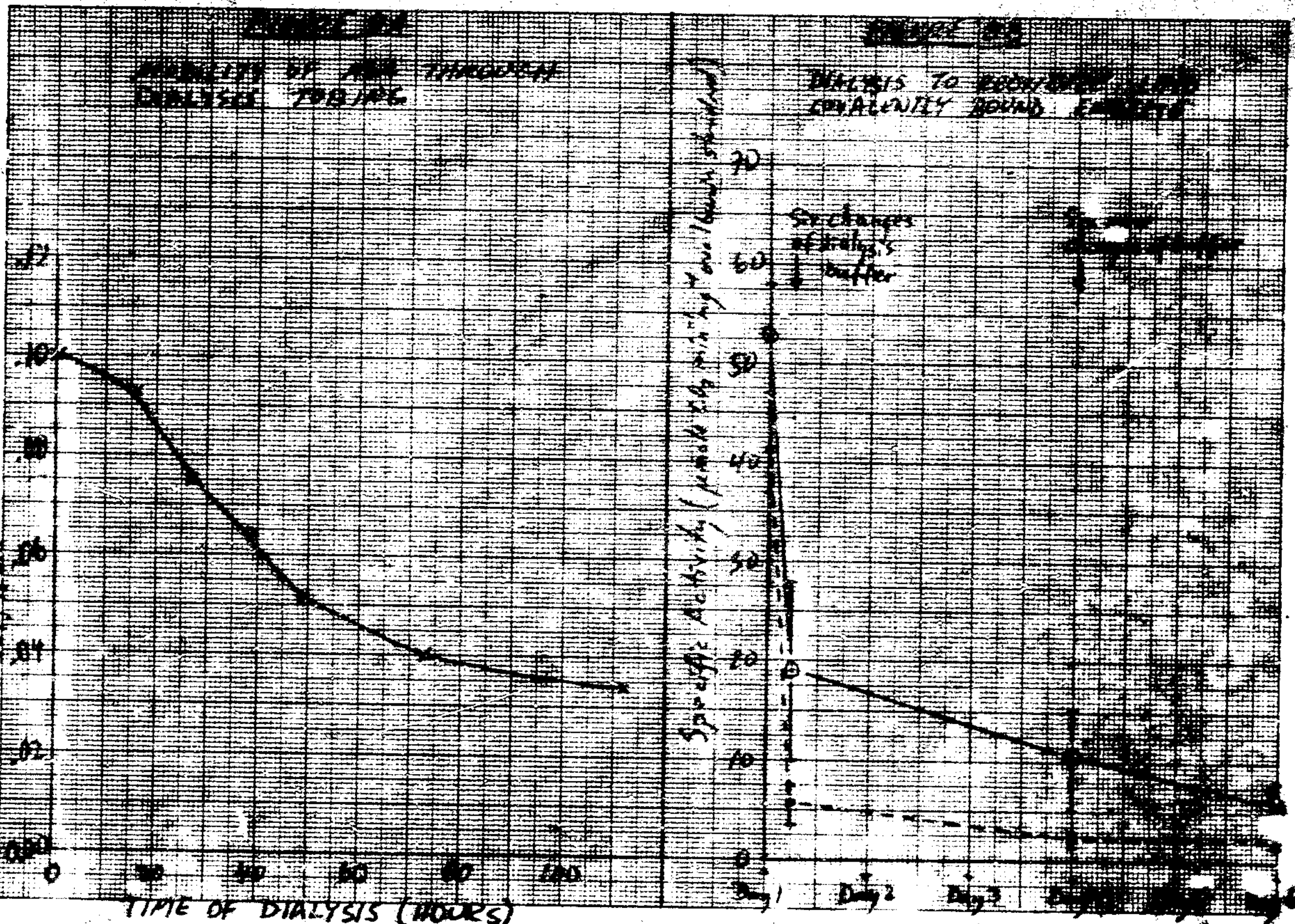


FIGURE 9

SPECTRA OF SAMPLES DIALYZED TO REDUCIBLE LIGAND, BY BOUND

Blank (10 kD Tubing) - 1:1 Buffer/H2O

Day 1 - after dialysis

Experimental SAMPLES

Control SAMPLES

2. Dithionite Reduction and Reoxidation with O_2

It seems as if I was able to reduce pyruvate oxidase in a covalent binding reaction mixture that didn't include ADA or carbodiimide. The control required 50 µl of 2 x 10^{-3}M Na dithionite to change the spectral curve, to one which suggested reduction. The enzyme concentration was .07 mg/ml. A cloudy solution containing white precipitate formed, confusing the spectral data. The precipitate could not be removed by centrifuging at 14,000 g for 20 minutes.

3. Chelation of Mn^{2+}

First it was established that ADA can be covalently bound to pyruvate oxidase in hepes buffer. Upon dilution, the activity of the experimental sample (containing carbodiimide) was 2.5 times as large as the control (lacking carbodiimide). After the solutions were incubated for an hour, EDTA was added. This did not seem to oxidize the sample with covalently bound ADA. The sample contained much white precipitate. In another run, EDTA was added only six minutes after the addition of carbodiimide. This seems to have reoxidized the sample, as seen by the spectra in figure 10. An unexplicable large peak was found in the 370-390 nm range. A slight yellowish coloring indicative of oxidized FAD was observed several minutes after the addition of EDTA. The sample was without any cloudiness, unlike the sample with lipid covalently bound to reduced pyruvate oxidase. Unfortunately, the EDTA treatment seems to have reduced the activity of the enzyme. The protein determination is based on ovalbumin standards of the Bio-Rad assay:

Figure 2.7 Recorded Signals of ADA Lovastatin by Chromatography

Sample	Specific activity (μmole $CO_2 min^{-1} mg^{-1}$ ovalbumin)
with EDTA treatment	6.15
without EDTA treatment	18.5

F. Spectral Studies with Anthroyloxydodecanoic Acid

The absorbance, excitation, and emission spectra of ADA were recorded in 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer. The spectra are shown in figures 11, 12 and 13. In all spectra the effects of the blank are accounted for by the position of the baseline.

Udenfriend and co-workers have shown that the fluorescent probe fluorescamine is a nonfluorescent molecule that forms highly fluoresecent primary amines upon covalent binding (16). Others have shown that α-parinaric acid increases in fluorescence intensity 40 fold upon binding to bovine serum albumin (17). Looking for similar enhancement effects upon binding, the fluorescence intensity of ADA in solution, noncovalently and covalently bound to pyruvate oxidase were measured. The concentration of ADA was the same in all three samples. The results are shown in figures 14A and 14B. The ADA:monomer ratio was 2:1. The experimental sample contained covalently bound ADA while the control contained noncovalently bound ADA. The experimental sample fluoresced more than the control, and both fluoresced less than ADA free in solution. The fluorescent peak of ADA free in assay buffer solution was actually 20% higher than shown. To fit the peak on the graph paper, I needed to decrease the fine sensitivity switch on the chart recorder amplifier. The emission peaks of the three samples in assay buffer occurred at a wavelength 15 nm lower than the emission peak of ADA in $NaPO_4$ buffer.

FIGURE 11

ABSORBANCE SPECTRUM OF ADA

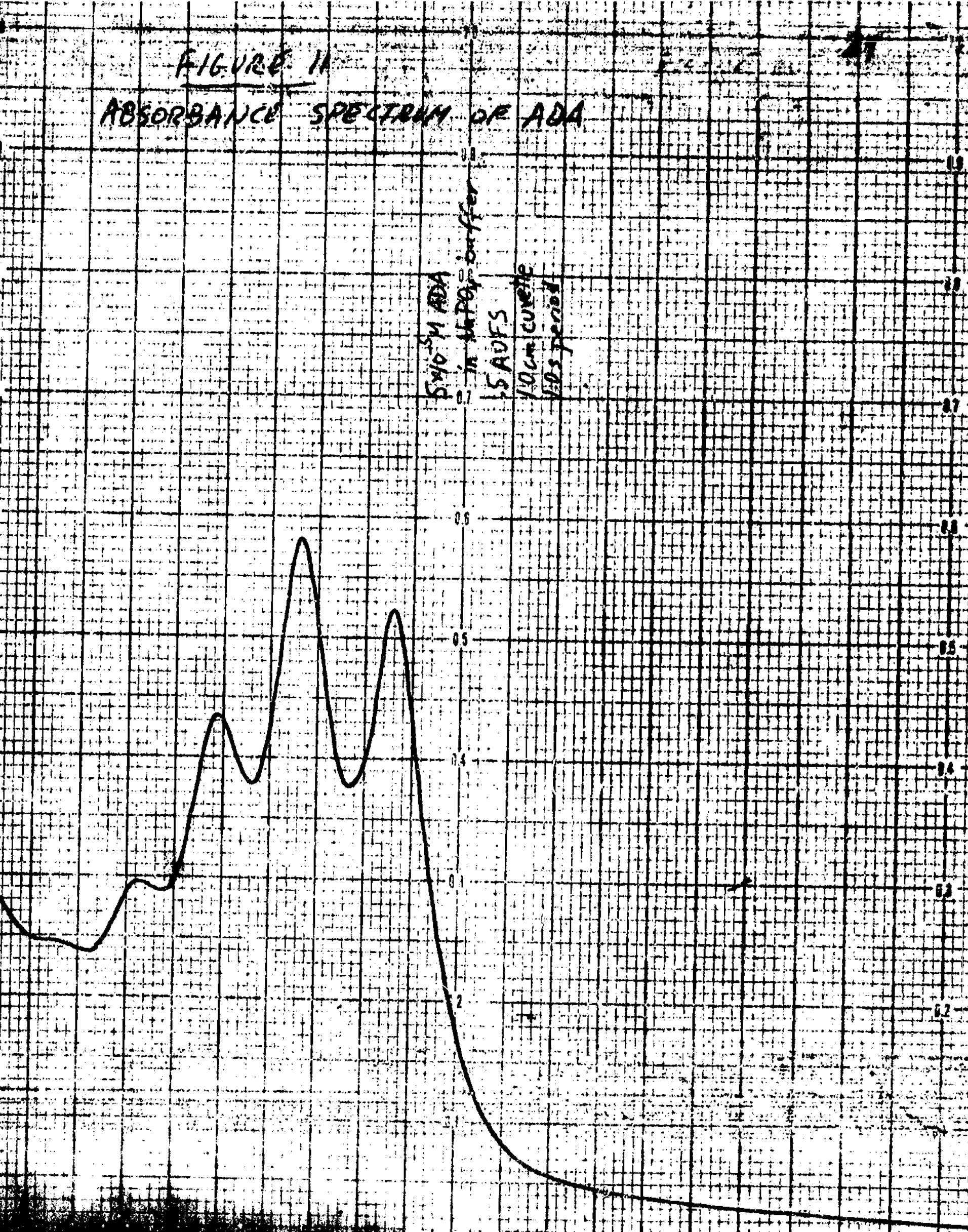


FIGURE 12

EXCITATION SPECTRUM OF ADA

$\lambda_{emission} = 460nm$

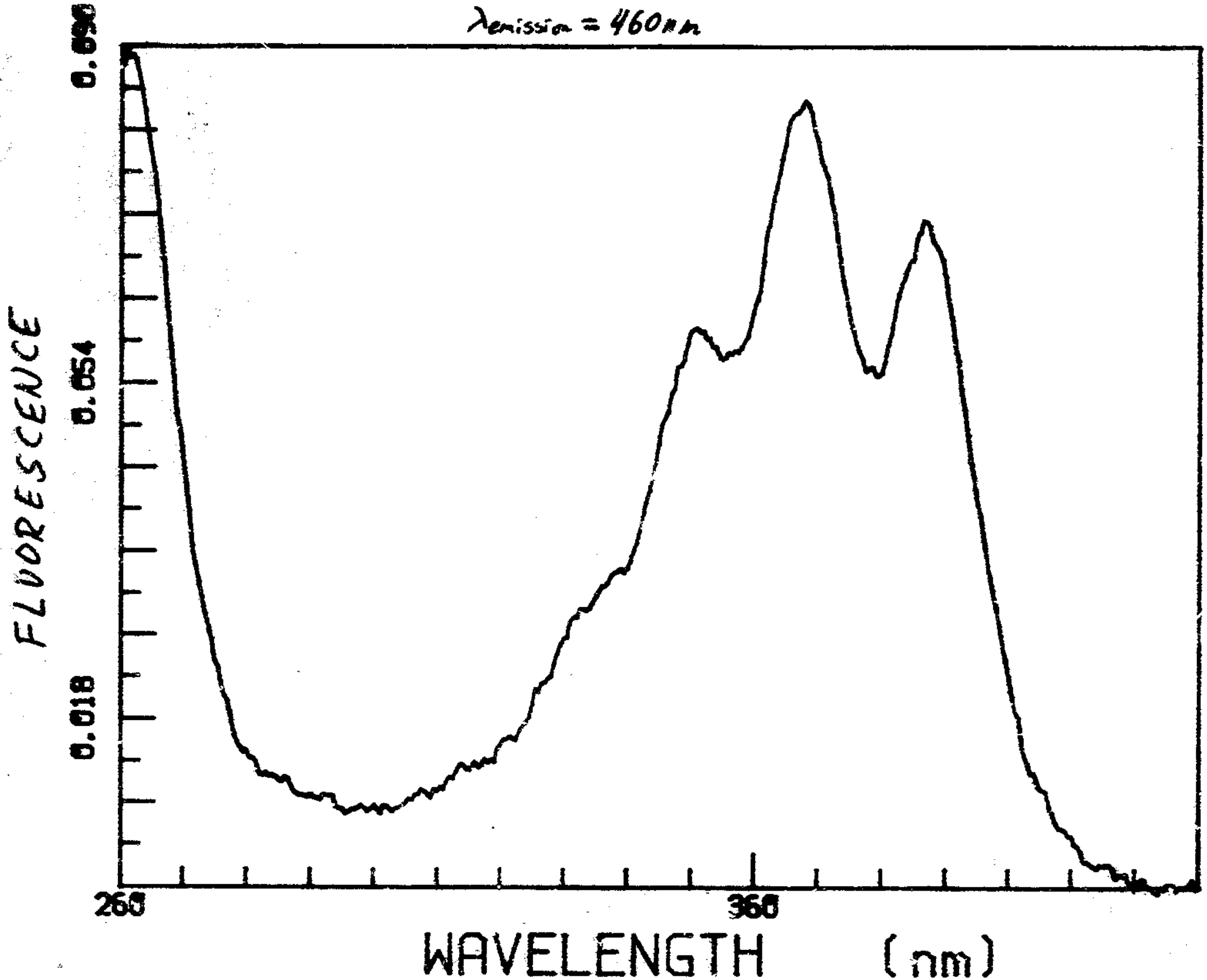


FIGURE 13

EMISSION SPECTRUM OF ADA

$\lambda_{excitation} = 361\,nm$

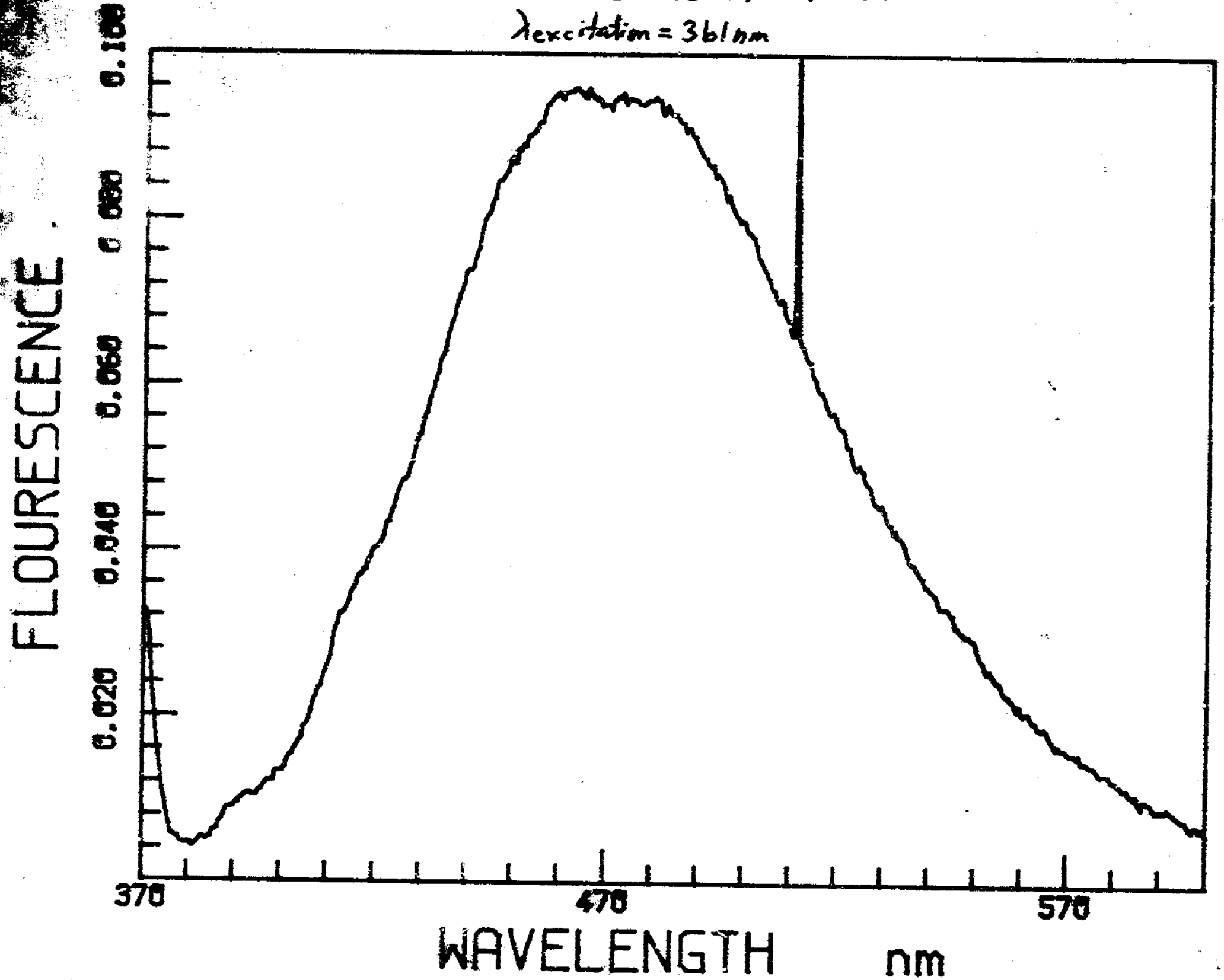


Figure 14A

FLUORESCENT EMISSION
OF COVALENTLY BOUND
ANS A

Excitation λ = 361 nm
Emission slit: 10 nm
Excitation slit: 10 nm
Uncorrected spectra

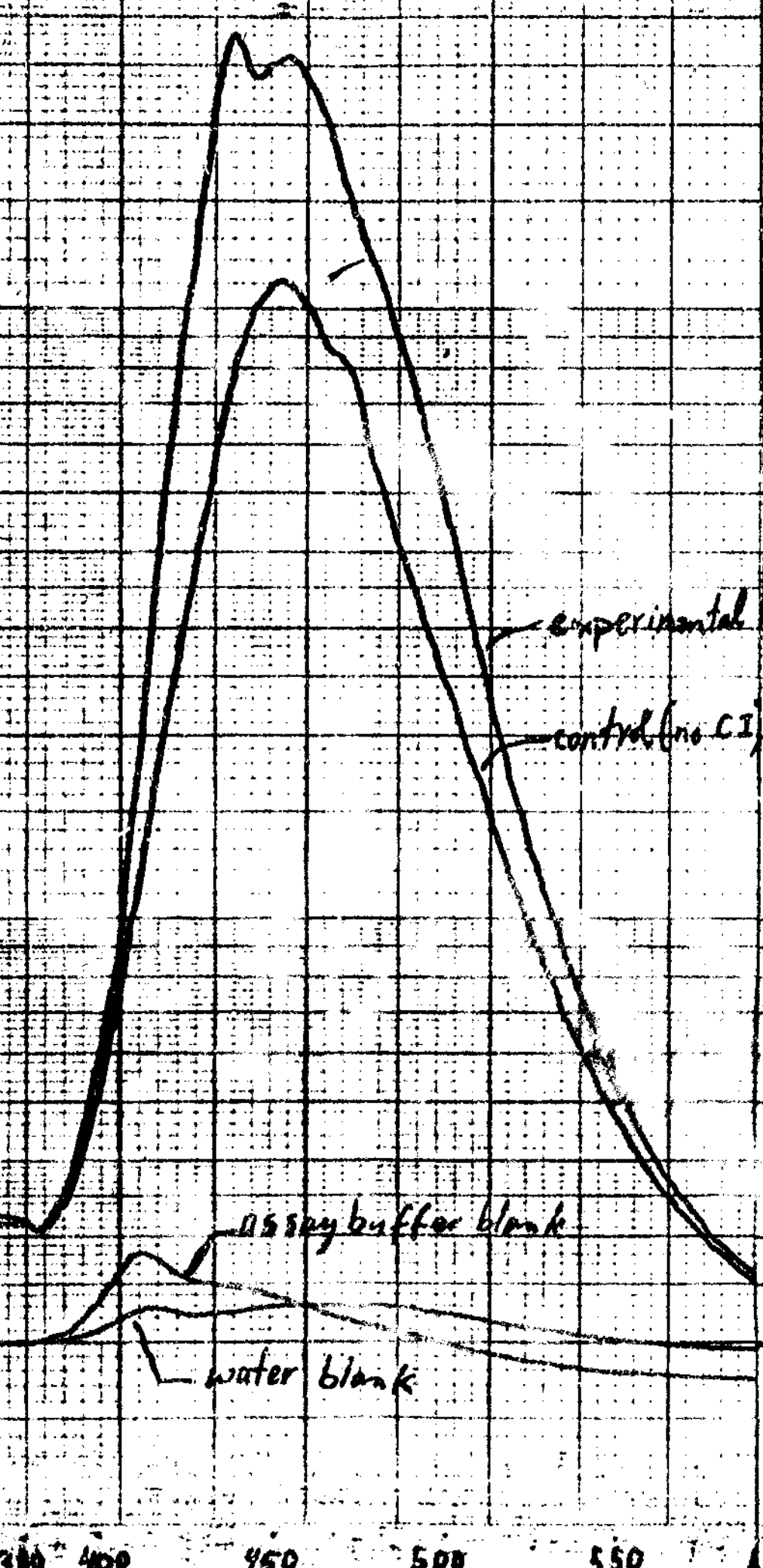
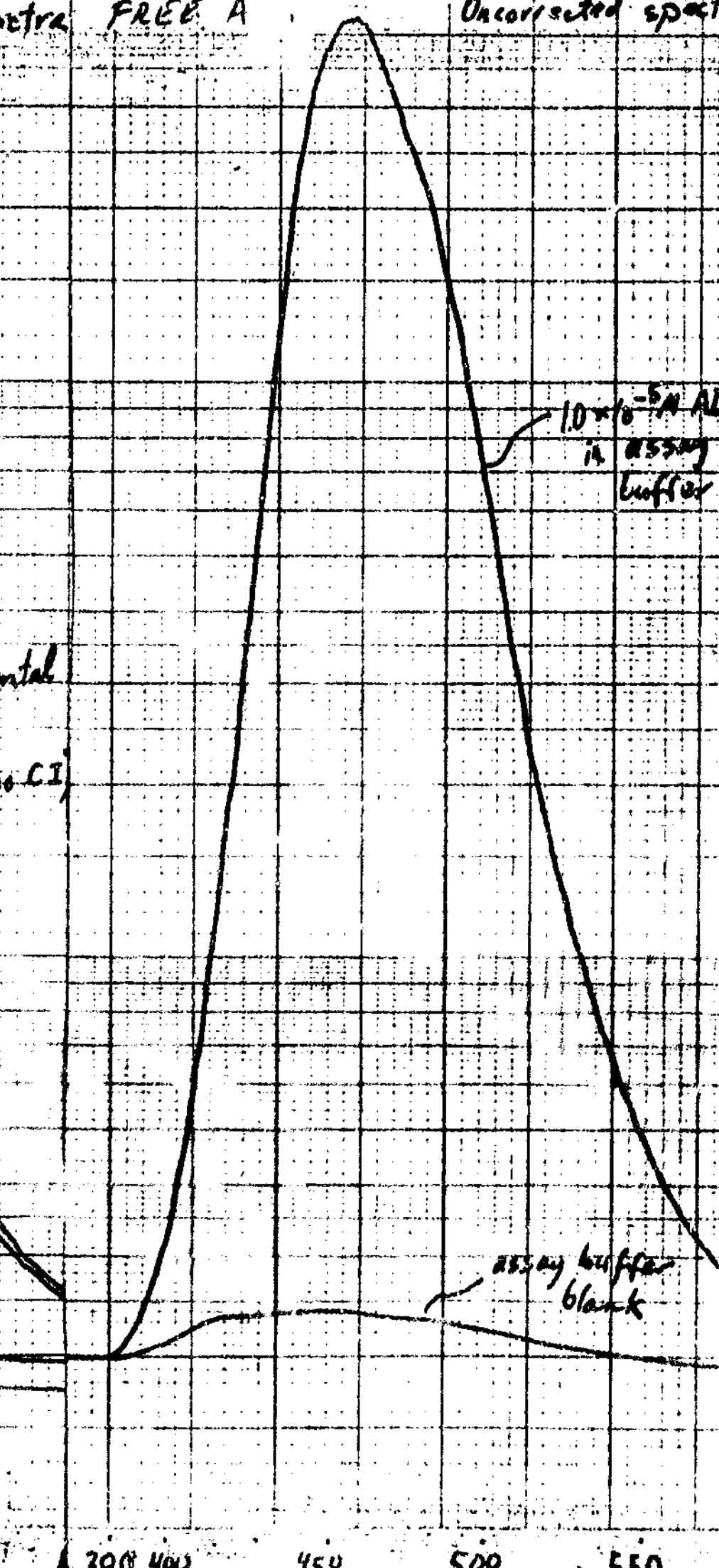


FIGURE 14B

FLUORESCENCE
EMISSION
FREE A

Excitation λ = 361
Emission slit: 10
Excitation slit: 10
Uncorrected spec



The three samples emitted maximally at 455 nm while the maximal emission of ADA in $NaPO_4$ buffer occurred at 470 nm.

The last fluorescence experiment attempted to determine whether energy transfer can be qualitatively observed. A reduced sample of ADA covalently bound enzyme was treated with EDTA to reoxidize pyruvate oxidase while another sample was treated with a water blank to keep the enzyme reduced. The ADA:monomer ratio was 2:1. The spectra of the samples are shown in figure 15. The unusually high fluorescence of the blank lacking ADA and EDTA was probably caused by probe contamination. The sample with EDTA turned a yellowish color upon EDTA treatment while the two controls were colorless. The EDTA treated sample was clear as water in the fluorescence cuvette, while the reduced sample was cloudy in the fluorescence cuvette.

G. Attempts to Separate Covalently Bound and Unbound Probe

1. Ammonium sulfate precipitation

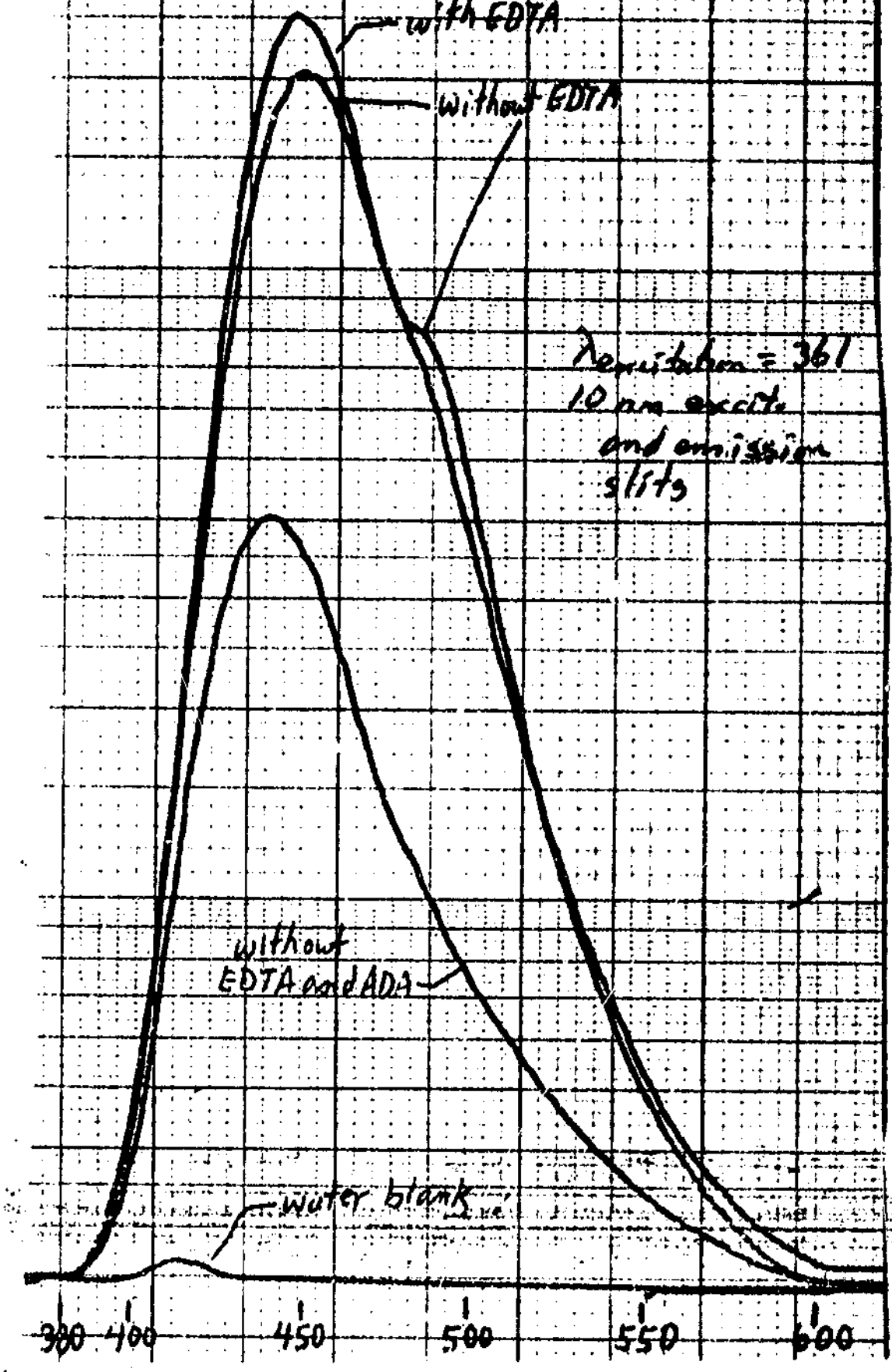
The following chart summarizes the results. The protein was determined by measurements of absorbance at 438 nm or 280 nm. The resuspended pellets containing the enzyme were cloudy white, suggesting that the enzyme was at least partially denatured.

	Total activity (μmole CO_2 min^{-1})		Total protein (mg)	
Sample	Exp.	Control	Exp	Control
Original	31.7	22.6	.58	.58
Final resuspension	7.4	3.2	3.2(1.5)	2.0 (.99)

The values in parentheses for the total protein determination are calculated from A_{280}.

FIGURE 15

FLUORESCENT EMISSION OF EDTA TREATED SAMPLE



2. Dialysis

The first experiment involved dialyzing a sample containing the reaction mixture used to covalently bind ADA and observing the fluorescence after two days of dialysis. Both the experimental and control samples showed a 90% loss in total activity after 24 hours of dialysis. Samples taken after 24 and 48 hours showed an emission peak at 461 nm. There was little or no difference observed between the emission peak of the experimental and control samples.

The second experiment tested the ability of ADA to leave the dialysis tubing. The slow mobility of ADA as measured by the loss in A_{367} of the dialysate is shown in figure 8A.

The third experiment was attempted to reoxidize lipid bound pyruvate oxidase. The results are given in chapter III E, section 1.

3. Ultrafiltration

The method was tried four times. Only once did I not get a substantial loss in total activity of both experimental and control samples. The protein concentrations were determined by absorbance at 438 nm. The spectra of the samples were measured after ultrafiltration. A gradually upward rising slope from 500 to 350 nm, as exemplified by the spectra of attempt #2, was typically observed. The spectra of attempt #2 are shown in figure 16. Such a spectral shape is compatible with reduced enzyme, but the absorbance was much too high for the maximal amount of enzyme present. Occasionally a yellow coloration of the ultrafiltration membrane was observed after concentrating. The results of the four attempts are:

350 nm

Control .5AUFS

Exp .5AUFS

400 nm

Exp .2AUFS

Exp .1AUFS

Control .1AUFS

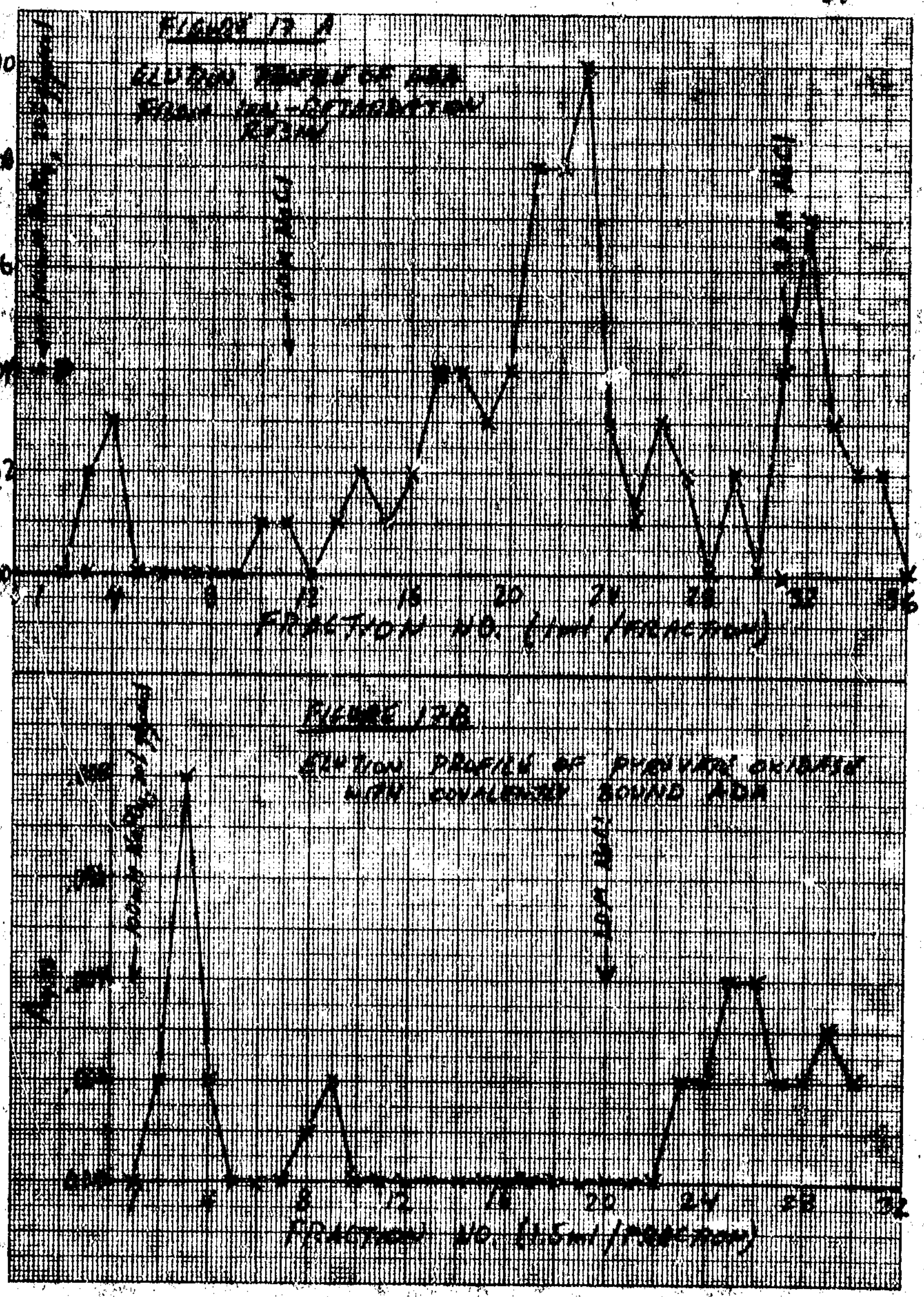
450 nm

Spectra of Lima Filtered Samples

	Total activity (μmole CO_2/min)		Total protein (mg)		Total times diluted and concentrated
Attempt #	Exp	Control	Exp	Control	
1 before ultrafil.	92.4	47.3	1.28	1.28	8
after ultrafil.	50.1	23.4	3.9	1.64	
2 before	25.4	11.7	.58	.58	125
after	5.2	1.26	1.5	.82	
3 before	18.3	---	.32	---	10
after	2.0	---	.28	---	
4 before	13.6	11.0	.26	.26	5
after	.1	0.0	.16	.085	

4. Ion-retardation resin

The elution profile of a sample of ADA in 100 mM $NaPO_4$, 20% glycerol, pH 6.0 buffer shows that most of the ADA stuck to the column until the high salt elution was begun. This is shown in figure 17A. The elution profile of pyruvate oxidase with covalently bound ADA shows a peak soon after the low salt elution was begun and another peak after the high salt elution. The elution profile of the first attempt with this column is shown in figure 17B. The activity of the enzyme covalently bound to ADA eluting in the first peak of the low salt elution was determined, as was the protein (by A_{438}) concentration:


.010
.008
.006
.004
.002
0.000
A367, Ion
Fraction No. (1 Sml/fraction)
A367, Ion
Fraction No. (1 Sml/fraction)

	Activity (μmole CO_2/min)		Protein (mg)	
Attempt	Exp	Control	Exp	Control
1. Sample loaded	16.4	5.5	.32	.32
Low salt peak	1.8	.10	.094	.031
2. Sample loaded	34		.64	
Low salt peak	1.7		.58	

All the samples exhibited a peak of A_{438} in the third fraction of the low salt elution.

5. Extraction with Organic Solvents

The loss in the specific activity with extracting ADA covalently bound enzyme samples with chloroform and heptane is shown in figure 18A. The protein was determined by the Bio-Rad microassay. In all the sample a white precipitate was noticed, especially after the mixing became more vigorous. The extraction of the ADA into chloroform is shown in figure 18B. The extent of extraction of ADA into heptane was not graphed because a standard sample of ADA in heptane could not be prepared.

6. Sephadex G-50 column

Reduced pyruvate oxidase in assay buffer was loaded on gel filtration columns of two sizes: 1 x 10 cm and 1.4 x 24 cm. The elution profiles are shown in figure 19 and 20. The peaks after the void volumes correspond to the elution of pyruvate oxidase. The peaks occurring later correspond to the elution of assay buffer components. The absorbance spectrum of fraction 5

FIGURE 2A

EFFECTS OF EXTRACTION ON THE SPECIFIC ACTIVITY OF NDA DEHALOGENASE BOUND CULTURE

C-●-C EXPERIMENTAL
●---● CONTROL (NO CI)
Δ-▲-Δ EXPERIMENTAL } INTO CELLS
▲---▲ CONTROL (NO CI) } INTO MEDIUM

SPECIFIC ACTIVITY (μmole Cl⁻/min-mg)

SAMPLE | VORTEX STIRRING | FASTER STIRRING | NUMBER OF SERIES OF HOMOGENIZER SHAKES

FIGURE 2B

EXTRACTION OF NDA INTO CELLS AND MEDIUM

C-●-C EXPERIMENTAL
●---● CONTROL

% NDA EXTRACTED

SAMPLE | GENTLE STIRRING | FURTHER STIRRING | HOMOGENIZER SHAKING

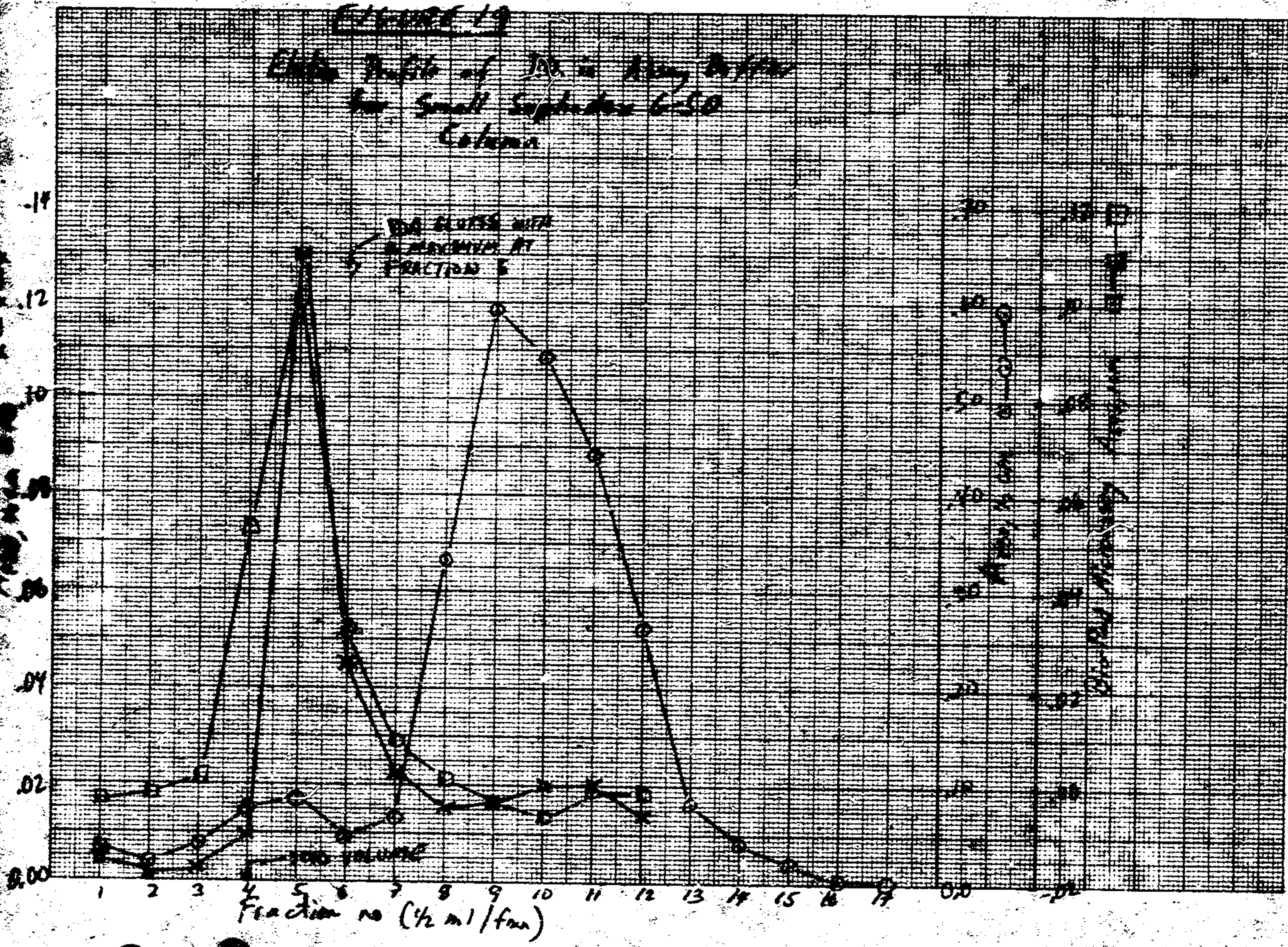
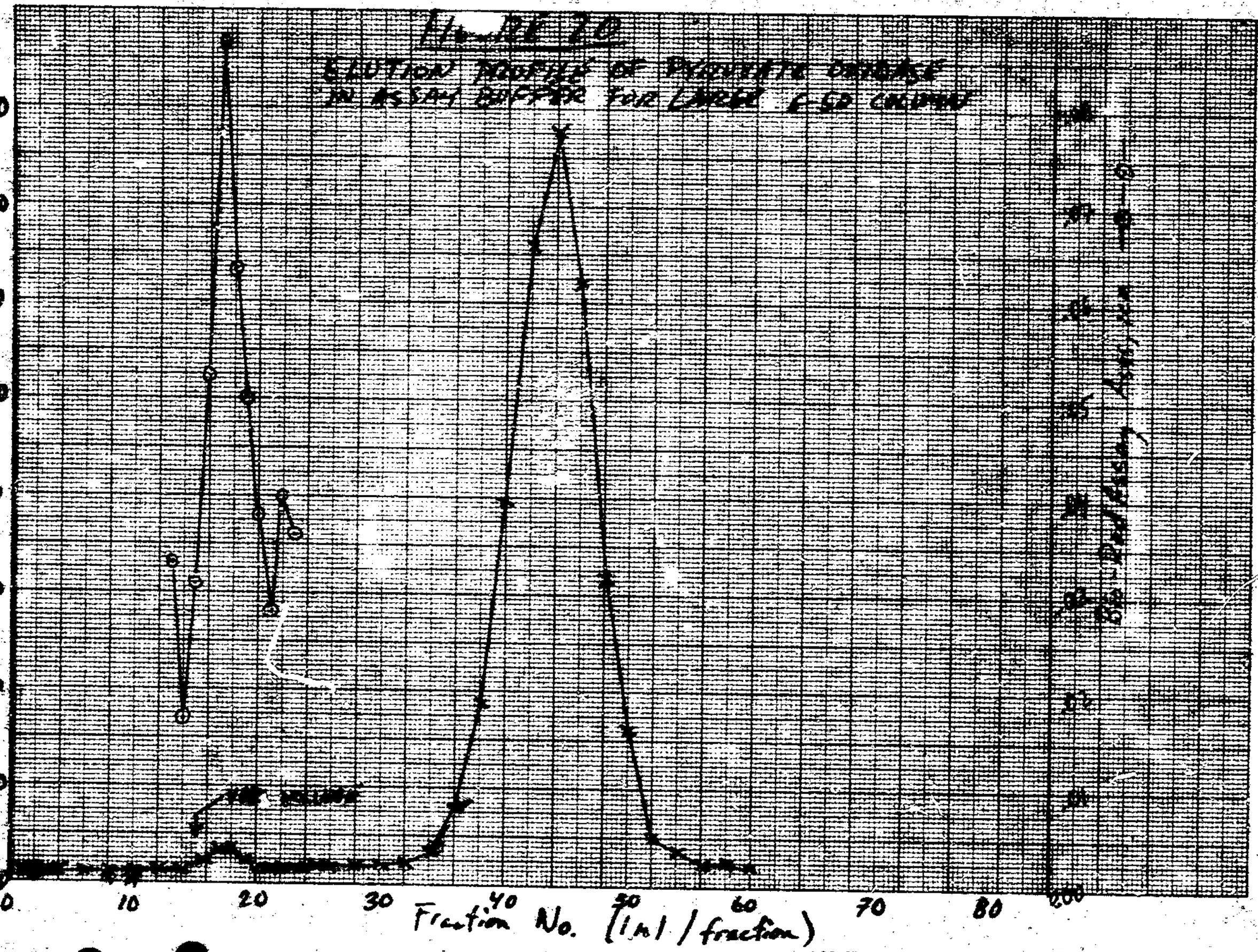


FIGURE 19
Column
VOID VOLUME
Fraction no (1/2 ml/frn)
.14
.12
.10
.06
.04
.02
0.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 20

ELUTION PROFILE OF PEPSINATE ONTOMAT
IN 4% SALT BUFFER FOR LARGE E-SD CRUDE



24

of the smaller column demonstrated an oxidized enzyme. The larger column was tried because ADA loaded on the smaller column eluted with a peak at fraction six. Thus, the overlap between the elution profiles of enzyme and ADA is too large for the smaller column. The protein yields were calculated by the Bio-Rad assay. The following yields were obtained from the columns:

Column	% Activity eluted in peak after void volume	% Protein eluted in peak after void volume
Small	50.	61.
Large	5.8	60.

IV. DISCUSSION

A. Purification of Pyruvate Oxidase

The purification technique worked well with the new CG-5 strain. Up to the low ionic strength precipitation step, I isolated nearly 70 mg of enzyme. Also, John Cronan's laboratory is developing a strain containing cloned pyruvate oxidase genes. This will increase the enzyme purification yield dramatically.

B. Probe Activation of Pyruvate Oxidase

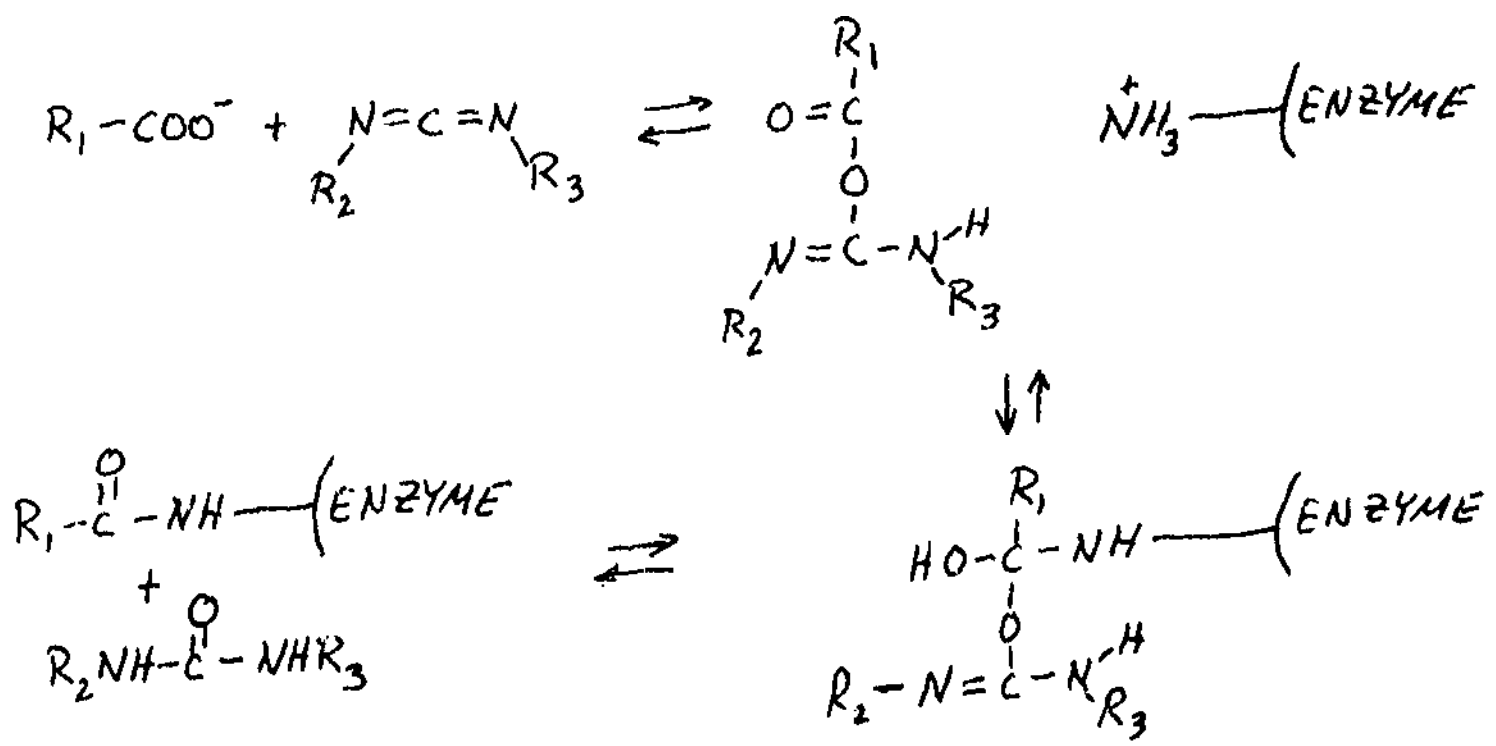
Since I could not covalently bind beta-parinaric acid to pyruvate oxidase I searched for other fluorescent probes that activate the enzyme. The activation profiles of four probes were tested. The profiles (figures 2 and 3) show that ADA and BPA activated the enzyme the most and ANS and PDA the least. Thus, I chose to work on covalently binding ADA instead of PDA or ANS to the enzyme. Because ADA contains a bulky anthracene group at the non-carboxy end of dodecanoic acid, I measured the effect of the anthracene moiety in activating the enzyme. Figure 3A shows that anthrance activated the enzyme only at high concentrations ($1x10^{-4}$ M). Since I typically used ADA concentrations around 10^{-5} M, I did not expect the enzyme to be activated much by the anthracene moiety of ADA.

The effects of DMSO on unactivated and lipid activated enzyme suggest DMSO's mode of action on the enzyme. DMSO's activation of unactivated enzyme and inactivation of lipid activated enzyme suggests that DMSO competes with the lipids for the enzyme's activation sites. Since DMSO does not activate the enzyme as much as the lipid does, the solvent seems to inactivate the SDS activated enzyme. The results also demonstrate that it is not correct to subtract the activation of the enzyme due to the DMSO control from the

activation of a lipid to obtain the solvent-corrected activation of the lipid. The final concentration of DMSO in the activation assays was 5% (v/v). Hence, DMSO reduced the lipid activation of the enzyme by only 5% in the activity assays for the lipids (figure 4B).

C. Covalent Binding of Lipids to Pyruvate Oxidase

The covalent binding reaction of a lipid to pyruvate oxidase was mediated by carbodiimide:



The dilation assay proves covalent binding between a fatty acid and the enzyme if the activity of the experimental sample increases with time while the activity of the control without carbodiimide remains at a lower activity and constant with time. This happens because diluting the samples will push the fatty acid binding equation towards the unbound state:

Enzyme + fatty acid ⇕ Enzyme-fatty acid complex

← Dilution favors

The covalently bound fatty acid will not dissociate upon dilution while the noncovalently bound fatty acid will dissociate upon dilution. The association state of the fatty acid-enzyme complex can be determined by the enzyme's activity.

The first fluorescent lipid I tried to covalently bind to pyruvate oxidase was beta-parinaric acid. I believe the results show that BPA was not covalently bound to the enzyme in these experiments. The strongest evidence for the lack of covalent binding is the small or no difference between the activity of the sample containing carbodiimide and the sample without carbodiimide in the dilution assay. Also, the poor activation of the experimental sample as % of the 20 μM SDS activity supports the conclusion that covalent binding did not occur. With ADA and lauric acid successful covalent binding, as demonstrated by the dilution assay, resulted in an activity of 50% and up of the 20 μM SDS activity. The average activation of the experiments with beta-parinaric acid resulted in only 30% activity of the 20 μM SDS activity.

I'm not sure why BPA did not covalently bind to the enzyme. It seems unlikely that BPA from two batches or pyruvate oxidase from two preparations were bad. I also tested and verified the purity of BPA by absorbance in solution. To test the reagents and my technique, I twice tried and succeeded in covalently binding lauric acid to the enzyme. Also, care was taken to deaerate all solutions coming into contact with BPA by argon bubbling.

Unlike with beta-parinaric acid, I have covalently bound anthroyloxy-dodecanoic acid to the enzyme. The optimal lipid:monomer ratio was 2:1 for a 5×10^{-6} M monomer concentration. The ratio is optimal because covalent binding was well demonstrated by the dilution assay and the low lipid:monomer ratio

would make removing unbound ADA an easier task than if a higher ratio were used. The removal of unbound ADA or nonspecifically and noncovalently bound ADA is necessary for accurate energy transfer experiments using ADA.

Figure 6B shows that maximal activation by covalent binding occurred at a 5:1 lipid:monomer ratio. Higher ratios, up to 40:1, did not change the activation significantly. The dilution assays with ADA exhibited the strange behavior that at lipid:monomer ratios of 5:1 or greater, both the experimental and control samples would show similar increases in activation with time. The increase in activation with time to an activation of 55% of the 20 μM SDS activity suggests that the enzyme associated with the lipid in such a way as to make the dilution assay ineffective. The critical micelle concentration of ADA suggests that this was the case. The CMC was determined to be 8×10^{-5} M while the concentration of the lipid in the samples exhibiting the strange dilution assay behavior began at 5×10^{-5} M. It seems probable that the enzyme associated with the ADA micelles so that the dilution of the micelle-enzyme complex did not dissociate the complex.

The last fluorescent lipid I tried covalently binding to pyruvate oxidase was pyrenedodecanoic acid. From the lack of a difference in the time dependent activation profiles of the experimental and control in the dilution assay and the low activation of the samples in the dilution assay, it does not appear as PDA was covalently bound to the enzyme. However, since the covalent binding reaction was run at very high lipid:monomer ratios, problems with micelles may have occurred, as with ADA. I did not attempt to work out the covalent binding reaction with PDA because ADA gave much better activation in the dilution assay.

D. Attempts to Reoxidize Reduced, Lipid Bound Pyruvate Oxidase

Several experiments were tried to switch pyruvate oxidase to the oxidized state after covalently binding ADA. Oxidized pyruvate oxidase does not aggregate whereas reduced enzyme aggregates. It is not known whether oxidized, lipid covalently bound pyruvate oxidase aggregates like the substrate reduced enzyme or forms clear solutions like the native, oxidized enzyme. Manipulations of the lipid covalently bound enzyme may be easier if the enzyme could be oxidized than if it were left in the reduced state. (The covalent binding reaction reduces pyruvate oxidase.) Also, energy transfer experiments must be conducted with a sample of the enzyme in the oxidized state. The three techniques that attempted to reoxidize the lipid covalently bound enzyme were:

1. Dialysis

Theoretically, the substrate pyruvate and the cofactor can be dialyzed out of the dialysate resulting in the reoxidation of the enzyme. Also, $K_3Fe(CN)_6$ added to the dialysis buffer should help reoxidize the enzyme. Dialysis was found not to be a good method to reoxidize the enzyme because of the immediate 2.5 fold loss in specific activity of the experimental sample. Precise energy transfer experiments cannot be made with a substantial amount of ADA covalently bound enzyme denatured. The spectra of the samples were inconclusive since the gradually upward sloping curve suggestive of reduced enzyme absorbed far too much for the known amount of enzyme in the solution. The large absorbance was probably caused by the cloudiness of the dialysate samples.

2. Dithionite Reduction and Reoxidation with O_2

I found this a clumsy method to try to develop. Since the following, more promising, method was suggested by M. Recny before further work on this method, I stopped working on it.

3. Chelation of Mn^{2+}

Mary Flannery has shown that chelating the divalent cation in a sample containing reduced pyruvate oxidase results in the inactivation of the enzyme upon mild proteolysis (18). Presumably the removal of the cation causes the enzyme to revert to its native, oxidized state that is inactivated upon mild proteolysis. The reduced form of pyruvate oxidase is activated upon mild proteolysis. As judged from the absorbance spectrum, the reduced, ADA covalently bound enzyme seems to have been reoxidized upon EDTA chelation. Unfortunately, there is a 66% loss in specific activity caused by this treatment. This technique may be further developed. The EDTA treated solution looked as a very promising solution to manipulate because it was clear as water. This is contrasted to the lipid covalently bound, reduced enzyme sample which was cloudy and contained small white precipitates.

E. Spectral Studies with Anthroyloxydodecanoic Acid

The similarity between the absorbance spectrum of ADA and fluorescence excitation spectrum were seen as expected. ADA is a good fluorescent probe to use in energy transfer experiments with the FAD cofactor. The overlap between the fluorescent emission of ADA and absorbance of FAD is large. Also, pyruvate oxidase fluoresces maximally at 340 nm while being excited maximally at 290 nm (19). Thus, there is no significant interference between the

fluorescence of ADA and pyruvate oxidase. ADA did not show an increase in fluorescence upon binding covalently or noncovalently to pyruvate oxidase. If the probe increased in fluorescence intensity 40 fold as cis-parinaric acid does upon binding to bovine serum albumin, the separation of covalently bound ADA and unbound ADA would not be necessary for accurate energy transfer experiments. But this was not found with ADA.

The last fluorescence experiment attempted involved looking for qualitative energy transfer between ADA and FAD. Positive results would have demonstrated qualitative energy transfer only, because the ADA:monomer ratio was 2:1, which is too high for the quantitation of energy transfer. The experiment involved comparing the fluorescence emission spectra of EDTA treated and untreated samples containing reduced, ADA covalently bound enzyme. If energy transfer occurred, the EDTA treated (oxidized) sample would fluoresce less than the untreated (reduced) sample because of the increased spectral overlap integral for the oxidized sample versus the reduced sample. No significant difference between the emission spectra of the two samples were observed. This may be caused by the confusion of the results or by the lack of energy transfer. The results could have been confused by too great a ratio of unbound to bound ADA or by the cloudiness of the EDTA untreated sample (the EDTA treated sample was clear). The lack of energy transfer could be due to too great a distance between ADA and FAD or, more likely, the loss of enzyme conformation caused by EDTA treatment.

F. Attempts to Separate Covalently Bound and Unbound Probe

To quantitatively measure energy transfer, the ADA that is not covalently attached to the enzyme must be removed from the solution. Six methods have been tried to accomplish this:

1. Ammonium sulfate precipitation

This technique should precipitate the ADA covalently bound pyruvate oxidase while the excess lipid remains in solution. It is a plausible technique because it is used successfully with native enzyme in the purification procedure. The results showed a drastic loss in total activity for both the experimental and control samples. I could not calculate the change in specific activity because the protein determinations by absorbance at 438 and 280 nm were not reliable. The unreliability is easily recognized by noticing that the total protein determined rose significantly. The correct way to have measured the protein values would have been by a method as the Bio-Rad, Lowry, or Biuret protein assay. In these assays, the protein determinations are not confused by cloudiness in the samples assayed.

Without information on the specific activities of the samples before and after the method, the effectiveness of the method cannot be absolutely determined. A loss in total activity, as I found in both samples, may be caused by the loss of enzyme or the loss of lipid from the solution. It may have happened that the experimental sample lost total activity because of a loss in the total enzyme while the control sample lost activity because of separation of lipid from the sample. Such a case would make the ammonium sulfate precipitation technique a possible way to separate bound from unbound ADA.

Even in light of the uncertainty, however, this technique seems not to be a promising technique to separate bound and unbound ADA. First, there is a 75% loss in the total activity of the experimental sample that must be due to the loss of enzyme, because the lipid was covalently bound to the enzyme. Second, white precipitate was observed in the resuspended samples, indicating

possibly the denaturation of enzyme. Third, G. Leisman has substantiated that ammonium sulfate precipitation of lauric acid covalently bound enzyme irreversibly denatures the enzyme (20). So in view of all the information available, I do not see this technique as promising.

2. Dialysis

Three experiments were tried to test the feasibility of dialyzing out unbound ADA from a dialysis mixture containing covalently bound ADA. The first involved measuring the fluorescent emission of the dialysate. Theoretically, the dialysate with unbound ADA will fluoresce less than the dialysate with covalently bound ADA because the unbound ADA can leave the dialysis bag while the bound ADA cannot. The results showed a 90% loss in total activity of both the experimental and control samples and little difference between the fluorescence of the two samples. When the experiment was suggested to me I had not optimized the lipid:monomer ratio for the dilution assay and did not realize that observing the fluorescence of the two samples is not a good way to show covalent binding has occurred. The problem is that the lipid:monomer ratio was 38:1. Thus, if the experiment worked or not, little or no difference in the fluorescence emission spectra would be expected.

The second experiment involved measuring the mobility of ADA through the dialysis bag. Since only half of the ADA moved out of the dialysis tubing after fifty hours of dialysis, the method is not a good way to separate unbound from covalently bound ADA.

The third experiment was undertaken to see if dialysis reoxidizes lipid covalently bound ADA. The large loss of specific activity of both the

experimental and control samples shown in figure 8B further demonstrate the unviability of dialysis to separate bound and unbound ADA.

3. Ultrafiltration

Theoretically, the unbound ADA should move through the ultrafiltration membrane while the covalently bound ADA should not because the enzyme cannot pass through the membrane. Before the technique was tried with covalently bound ADA, experiments showed that ADA did not pass through the membrane, but stuck to it.

From the results with covalently bound ADA, the absolute effectiveness of the ultrafiltration technique cannot be determined. The problem is that I used absorbance at 438 nm to determine the protein concentration. This is an unreliable method to use with any solution but pure, oxidized enzyme solutions. When the results are combined with other information, the method does not seem to be the optimal way to separate bound and unbound ADA. Stevens and Gennis have observed that substrate reduced enzyme at concentrations of greater than 0.3 mg/ml aggregated and precipitated (21). I have observed a loss of 50 to 100% of total activity of the experimental samples using the ultrafiltration method. The use of the ultrafiltration to concentrate the native, oxidized enzyme in the purification procedure often results in a loss of 40% of the total activity. Thus, the enzyme, particularly the lipid bound, reduced form, appears to be too sticky for the ultrafiltration technique to be very successful. Additionally, the confusing spectra of the ultrafiltrated samples are probably caused by the cloudiness of the samples. Accurate fluorescence measurements cannot be made with cloudy samples.

4. Ion-retardation resin

Kapp and Vinogradov have devised a quick method to remove SDS from proteins (22). They showed that loading a sample with a 400 molar excess of SDS over ovalbumin onto an ion-retardation AG11A8 column resulted in the elution of a 1:0.6 molar ratio of SDS to ovalbumin. The lipid stuck to the column and the protein eluted under low salt elutions. I have found that ADA sticks to the column, but I could not quantitate the protein yield of pyruvate oxidase covalently bound to ADA. The protein concentration of the elemants was measured by A_{438}, a technique that is not accurate with enzyme not known to be pure and oxidized. However, this technique to remove unbound ADA from covalently bound ADA does not seem promising. The activity of the lipid covalently bound enzyme eluting in the A_{438} peak of the low salt wash was only 5-10 % of what was loaded. Some enzyme eluted after the high salt wash, but that enzyme is useless since ADA was shown to elute after the high salt wash.

5. Extraction with Organic Solvents.

The large loss in specific activity of the samples with extraction indicates that extraction of ADA with chloroform and heptane is not a good technique to separate covalently bound and unbound ADA. Significant amounts of ADA were extracted into the chloroform phase only after a loss of at least 50% of the specific activity.

6. Sephadex G-50 column

The results of the column runs were encouraging. Substrate reduced enzyme was oxidized and separated from the substrate and cofactors by running the enzyme through a 10 cm column. The activity and protein yields were

high. A 24 cm column was prepared because ADA eluted too closely to the enzyme in a control run. Assay buffer reduced enzyme eluted through the larger column inactivated. This was not expected, since the smaller column worked in eluting active enzyme. Although the initial results here are encouraging, it should be noted that others have encountered technical problems with eluting reducing pyruvate oxidase from Sephadex columns. Stevens and Gennis reported that substrate reduced enzyme will not elute from Sephadex, Sepharose, Bio-Gel, and Ultrogel gel filtration media (21). Also John White attempted to separate unbound lauric acid from covalently bound lauric acid using a Sephadex G-10 column. His results wavered. He finally seemed to think that the enzyme stuck to the column.

G. Theory of Energy Transfer Applied to Pyruvate Oxidase

Although I have not attempted quantitative energy transfer experiments, I think it would be helpful to outline the theory as applied to this project. The efficiency of energy transfer can be measured by three methods:

a) From the change in the excited state lifetime of the energy donor with and without the presence of the energy acceptor
b) From the change in the quantum yield (or relative fluorescent intensity) of the energy donor with and without the presence of the energy acceptor
c) From the excitation spectrum of the energy acceptor.

The efficiency of energy transfer, $E = \frac{r^{-6}}{r^{-6}+R_0^{-6}}$

where $R_0 = (JK^2Q_0n^{-4}) \times 9.7 \times 10^3$ Å

and J = spectral overlap between the absorbance spectrum of the energy acceptor and the emission spectrum of the energy donor

K^2 = orientation factor

Q_0 = quantum yield of the donor in the absence of the acceptor

n = index of refraction (11).

In this system, the fluorescent lipid, ADA, would be the energy donor and the FAD cofactor would be the energy acceptor. With the FAD cofactor as the energy acceptor, energy transfer can only be measured by a) and b), since FAD does not fluoresce in the enzyme. However, if FMN or Riboflavin could be substituted for FAD with pyruvate oxidase maintaining activity, fluorescence of the flavin group may be observed since the quenching adenine moiety of FAD would not be present. This would enable all three methods to be used for energy transfer experiments.

The spectral overlap constant, J, should be large with ADA and FAD. ADA emits maximally at 455 nm in assay buffer while FAD absorbs maximally at 438 nm. Q_0 must be determined by first covalently binding ADA to the enzyme and subsequently removing the FAD cofactor, perhaps by a procedure similar to one developed to remove FAD from native enzyme (12). The orientation factor introduces the largest error in energy transfer experiments. The factor can be determined by the emission anisotropy of the donor. Stryer claims, for a

typical value of emission anisotropy, the error introduced in measuring a distance of 40 A between chromophore centers is 20% (11). Last, Koland and Gennis have applied energy transfer methods to determine the distance between FAD and the TPP binding site in pyruvate oxide. Because of difficulties in determining the orientation factor and Q_0, they could only establish an upper limit of 20 A as the distance between the two sites (23).

V. CONCLUSION

The purpose of this project was to determine the distance between the fatty acid high affinity binding site and the FAD cofactor of pyruvate oxidase. Fluorescence energy transfer between a fluorescent fatty acid and the FAD cofactor was to be utilized. Although I have not been able to run quantitative energy transfer experiments, I have worked through several preliminary steps towards the goal.

Pyruvate oxidase was shown to be activated by four fluorescent probes. They include beta-parinaric acid, anthroyoxydodecanoic acid, pyrenedodecanoic acid, and anilinonophthalenesulfonic acid. The covalent binding reaction and subsequent dilution assay demonstrating covalent binding was worked out for ADA. The covalent binding reaction could not be worked out with BPA. I did not attempt to work it out with PDA or ANS because ADA activated the enzyme more than PDA or ANS.

ADA is a good probe for energy transfer studies with FAD. The spectral overlap between ADA and FAD is large. The dilution assay worked optimally with a 2:1 ADA:monomer ratio. This is advantageous because only a low amount of unbound ADA need be removed from the covalent binding reaction mixture to perform accurate energy transfer experiments. Also, ADA at such a low concentration does not form a cloudy solution, which could affect fluorescence measurements. ADA has the advantage over BPA that solutions containing ADA need not be deaerated as solutions containing BPA do.

Three methods were tried to reoxidize ADA covalently bound enzyme. Dialysis and reduction by dithionite were found ineffective or clumsy. Chelation of Mn^{2+} was found to be a promising method. Treatment by EDTA seemed to have reoxidized the enzyme but also caused some inactivation. The

EDTA treated sample was clear, unlike the untreated sample containing reduced, ADA covalently bound enzyme.

Six methods to separate covalently bound ADA from unbound ADA were tried. Precipitation by ammonium sulfate, ultrafiltration, dialysis, chromatography by ion-retardation resin, and extraction with chloroform and heptane were either demonstrated ineffective or shown not to be promising. Chromatography by G-50 Sephadex gave contradictory, but promising results. Separation of bound and unbound ADA was shown to be a necessary step for energy transfer experiments, since ADA covalently bound to the enzyme did not increase in fluorescence intensity over free ADA. Finally, the general scheme of energy transfer applied to pyruvate oxidase was worked out.

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